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The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

Letter from the Editors ---------- 1

Clinical and Experimental

Early History of the Central Society for Clinical Research. Lawrence D. Thompson, M.D., St. Louis, Mo. ---------- 3

Teachers of Medicine. W. Barry Wood, Jr., M.D., St. Louis, Mo. ---------- 6

The Relation of Life Stress and Emotions to Human Sebum Secretion and to the Mechanism of Acne Vulgaris. Thomas H. Lorenz, M.D., David T. Graham, M.D., and Stewart Wolf, M.D., New York, N. Y. ---------- 11

An Evaluation of the Measurement of the Cardiac Output and of the So-Called Pulmonary Blood Volume by the Dye-Dilution Method. Joseph T. Doyle, M.D., Joseph S. Wilson, M.D., Charles Lepine, M.D., and James V. Warren, M.D., Atlanta, Ga. ---------- 29

Effect of Prolonged Administration of Radioactive $Zinc^{65}$ on the Pancreas. James W. Sherrill, M.D., and Arne N. Wick, Ph.D., La Jolla, Calif. ---------- 40

Excretion of Water and Electrolytes in Patients With Essential Hypertension. William A. Brodsky, M.D., Louisville, Ky., and Cincinnati, Ohio, and Harry N. Graubarth, M.D., Cincinnati, Ohio ---------- 43

The Use of Cobaltous Chloride in the Anemia Associated With Chronic Renal Disease. Frank H. Gardner, M.D., Boston, Mass. ---------- 56

Maintenance Therapy of Pernicious Anemia With Vitamin B_{12}. Gordon C. Meacham, M.D., and Robert W. Heinle, M.D., Cleveland, Ohio ---------- 65

The Combined Anticonvulsant Activity and Toxicity of Dilantin and N-Methyl-5-Phenylsuccinimide. Graham Chen, Sc.D., M.D., and Charles R. Ensor, M.S., Detroit, Mich. ---------- 78

Post-Transfusion Survival of Erythrocytes Stored in a Solution of Ethylenediamine Tetraacetic Acid and Dextrose. Charles C. Sprague, M.D., John B. Shapleigh, M.D., Sue Mayes, B.S., Robert D. Lange, M.D., and Carl V. Moore, M.D., St. Louis, Mo. ---------- 84

Variation in Complement Fixation Antigen Production by Different Strains of Histoplasma Capsulatum Grown on Two Media. Joseph Schubert, Ph.D., Libero Ajello, Ph.D., Sarah Stanford, A.B., and Virginia Q. Grant, A.B., Atlanta, Ga. ---------- 91

An Evaluation of the Middlebrook-Dubos Hemagglutination Test. Edward F. Rabe, M.D., and William S. Spicer, Jr., M.D., Kansas City, Kan. ---------- 98

(Contents continued on inside front cover)

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Contents—(Continued from front cover)

The Migration and Oxidation-Reduction Activity of Leukocytes From Patients With Acute Disseminated Lupus Erythematosus. S. N. Chaudhuri, M.B.B.S., and Samuel P. Martin, M.D., Durham, N. C. ---------- 108

The Role of Cold Hemagglutinins in Frostbite. Capt. David Weiner, Medical Corps, United States Army ---------- 114

On the Duration of Penicillin Action in Relation to Its Concentration in the Serum. Harry Eagle, M.D., Ralph Fleischman, and Mina Levy, A.B., Bethesda, Md. ---------- 122

Clinical Laboratory Studies in Experimentally-Induced Epidemic Nonbacterial Gastroenteritis. Irving Gordon, M.D., John K. Meneely, Jr., M.D., Gordon D. Currie, M.D., and Alice Chicoine, Albany, N. Y. ---------- 133

Prevention of Paralytic Poliomyelitis in Tonsillectomized Cynomolgus Monkeys by Human Gamma Globulin. J. M. Adams, M.D., R. A. Boak, M.D., C. M. Carpenter, M.D., J. D. French, M.D., S. J. Klein, Ph.D., J. J. Pressman, M.D., and J. L. Smith, M.D., Los Angeles, Calif. ---------- 142

Recent Changes in Sensitivity of Micrococcus Pyogenes to Various Antibiotic Agents. Gerald M. Needham, Ph.D., and Donald R. Nichols, M.D., Rochester, Minn. ---------- 150

Laboratory Methods

Quantitative Determination of Nitrate by Enzymic Reduction to Nitrite. William R. Straughn, M.S., Inez Greene Travis, M.S., and Edwin P. Hiatt, Ph.D., M.D., Chapel Hill, N. C. ---------- 157

Identification of Abnormal Hemoglobins by Means of Paper Electrophoresis. Theodore H. Spaet, M.D., San Francisco, Calif. ---------- 161

An Accumulating Impulse Counter. Akira Horita, M.S., and James M. Dille, Ph.D., M.D., Seattle, Wash. ---------- 166

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CLAYTON G. LOOSLI, M.D.

Associate Editor
MATTHEW BLOCK, M.D.

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950 E. 59th Street
Chicago 37, Illinois

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LETTER FROM THE EDITORS

THE Editors and members of the Editorial Board wish to take this opportunity to express their appreciation and thanks for the help they have been given by their colleagues and many other individuals in other institutions in providing critical appraisal of manuscripts submitted to the JOURNAL for publication. The individuals whose names are listed below have rendered valuable editorial assistance during the past year.

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As of Dec. 30, 1952, Dr. Charles A. Doan, Dr. Wesley W. Spink, and Dr. Cecil J. Watson, after serving two three-year terms, will retire from the Editorial Board. The Editors take this opportunity to thank them for their valuable help and to welcome Dr. Richard B. Capps, Dr. Wendell H. Hall, and Dr. Richard W. Vilter who will replace them on the Board for the three-year term beginning Jan. 1, 1953.

The Editors would like to call attention to the fact that our Journal pages for manuscript publication are limited. Therefore, every effort should be made by authors submitting papers to avoid duplication of data in the text, tables, and figures. Young investigators inexperienced in preparing manuscripts tend to err in this respect. Often valuable assistance can be obtained from their senior associates in preparing data in a clear, concise manner. Likewise, it seems to the Editors that established investigators having responsibility for training young men could contribute to their development immeasurably by critically reviewing their manuscripts before they are submitted for publication.

This JOURNAL prefers manuscripts prepared under the following headings: Introduction, Materials and Methods, Results, Discussion, and Summary. Academic degrees of the authors and complete bibliographies are required. When these are incomplete there is, of necessity, a delay in processing the manuscripts for publication. For detailed information concerning the preparation of manuscripts the authors are referred to the Editorial Communications page usually found on the inside of the back cover of the JOURNAL.

—*Clayton G. Loosli, M.D.*
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—*Matthew Block, M.D.*
Associate Editor

EARLY HISTORY OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

LAWRENCE D. THOMPSON, M.D.

ST. LOUIS, MO.

THE organization meeting in Rochester, Minn., on April 20-21, 1928, obviously was not the true beginning of the Central Society for Clinical Research. Such organizations have their inception in the mind of some individual who is aware of a pressing need, and who also can picture in his mind a satisfactory means of meeting that need or demand.

World War I had been the impetus which had started a tremendous surge in Clinical Research and Investigative Medicine in all its branches. The number of societies for the presentation of the results and progress of Clinical Research was sharply limited. This limitation was also marked by geographic bounds. Journals for the publication of such data were months and even years behind schedule. This need had been met, partially, in the eastern portion of the United States by the American Society for Clinical Investigation, which had grown out of the much older Association of American Physicians. It was extremely difficult for a young man from the Middle West to present his work before these existing organizations. On the other hand, the number of young men in the Middle West medical centers who were engaged in clinical and basic science research problems was rapidly increasing.

In December, 1919, a group of established clinicians from the Middle West medical centers of Chicago, Iowa City, Minneapolis, Rochester, and St. Louis, men who were in a large part members of the Eastern societies, banded together and organized the Central Interurban Clinical Club. (Chicago, Ill.—Dr. Frank Billings, Dr. Joseph A. Capps, Dr. James B. Herrick, Dr. Ernest E. Irons, Dr. Joseph L. Miller, and Dr. Rollin T. Woodyatt; Iowa City—Dr. Campbell P. Howard; Minneapolis—Dr. S. Marx White; Rochester, Minn.—Dr. Henry S. Plummer and Dr. Leonard G. Rowntree; St. Louis, Mo.—Dr. George Dock and Dr. Canby Robinson.)

The objects of this Society were listed as follows: (A) the stimulation of original work and investigation; (B) the development and improvement of methods of teaching; (C) demonstrations of work in "borderline" subjects; (D) a better knowledge of the men, methods, and work of other clinics; (E) the promotion of good fellowship among the members of the Club.

Informal meetings were held twice a year, and the various clinics were visited in rotation. The membership of this organization was slowly increased in number. The problem of gaining admission to the established Societies for Clinical Investigation confronting the young men in these clinics was appreciated by the members of this group.

In November, 1920, Dr. Billings discussed this matter before the Club and was instructed to present the subject again at subsequent meetings. Although not so stated in the minutes, it seems likely that the idea might have originated with Dr. Billings. Substantiating this assumption is the fact that no further discussions along this line were recorded in the minutes of the next four meetings. For some reason Dr. Billings was absent from these meetings. At the meeting in June, 1923, Dr. Billings again discussed: "the organization of a new society." There apparently was further discussion of the project at

the next two or three meetings. In November, 1924, it was decided to organize a Club, similar to their own, for the younger men of the schools represented. It was thought that such a club of younger men would aid in the organization of a larger Society for Clinical Research. As a fruition of these ideas, two young men from each of the centers of Chicago, Iowa City, Minneapolis, Rochester, and St. Louis were invited to meet with the Central Interurban Clinical Club in Rochester on Dec. 5, 1925. (Chicago—Dr. R. W. Keeton and Dr. W. H. Nadler; Iowa City—Dr. Lee C. Gatewood and Dr. Frank J. Rohner; Minneapolis—Dr. C. A. McKinlay and Dr. Jay A. Myers; Rochester—Dr. George E. Brown and Dr. Fredrick A. Willius; St. Louis—Dr. A. P. Briggs and Dr. L. D. Thompson.) At this time, under the guidance of the parent Club, the Central Clinical Research Club was formed. Thus, the first step in the plan for a larger Society for Clinical Research was accomplished.

The project of a new Society for Clinical Research was the main topic of discussion at the respective meetings of both Clubs until the fall of 1926. At this time a joint committee was appointed to proceed with plans for the organization of such a new society. (Central Interurban Clinical Club—Dr. N. C. Gilbert, Dr. W. H. Olmsted, Dr. Leonard G. Rowntree; Central Clinical Research Club—Dr. George E. Brown, Dr. Norman M. Keith, Dr. Fredrick A. Willius).

Dr. Rowntree was instructed, "To write a letter on behalf of the Central Interurban Clinical Club to the Secretary of the American Society for Clinical Investigation, stating that it is our intention to further such a Society in the Middle West, and that he would like to cooperate with the parent Society (American Society for Clinical Investigation) in every way." At the meeting of the Central Interurban Clinical Club of Dec. 3, 1927, which Dr. Willius, representing the Central Clinical Research Club, attended as a guest, the following report was made: "Dr. Rowntree stated that the foundation of a new Society had the best wishes of the American Society for Clinical Investigation, but that the latter did not wish to participate actively in its formation." At this same meeting it was determined to have a joint meeting with the Central Clinical Research Club in the spring of 1928 and to invite to this meeting a number of representative men from the Middle West. The two Clubs, together with the invited men, would then organize a new Society for Clinical Investigation. There is no accurate list of the men invited to attend this meeting. The Council members of the respective Clubs and the members of the joint committee had combined to form the list and invite the men to attend.

The joint meeting of the two Clubs and their many guests took place in Rochester, Minn., on April 20-21, 1928. At an afternoon gathering on April 20, 1928, Dr. Willius presented the objects of the meeting, and the idea of a Society for Clinical Investigation was outlined in greater detail by Dr. Rowntree. A proposed constitution for such a society was read. There followed a general discussion of the idea which was participated in by many men from various medical centers. No action was taken at this time, however, and the meeting was adjourned until the next afternoon.

At 2:00 P.M., on April 21, 1928, the Clubs and their guests again assembled. By acclamation, Dr. Fredrick A. Willius was appointed temporary Chairman, and Dr. L. D. Thompson temporary Secretary. The prepared constitution, changed only in minor details from that presented the afternoon before, was read and officially adopted. The name "Central Society for Clinical Research" was decided upon from the many suggested. Thus, the actual organization of the Central Society for Clinical Research was accomplished. A nominating

committee presented the following names for officers of the Society and they were duly elected: *President,* Dr. Frank Billings, Chicago; *Vice-President,* Dr. Leonard G. Rowntree, Rochester; *Secretary,* Dr. L. D. Thompson, St. Louis; *Treasurer,* Dr. William S. Middleton, Madison, Wisconsin; *Councilors,* Dr. Ernest E. Irons, Chicago, Dr. George E. Brown, Rochester, and Dr. Roger S. Morris, Cincinnati; *Alternates,* Dr. Robert W. Keeton, Dr. Horace M. Korns, and Dr. Russell L. Haden.

Dr. Franklin C. McLean invited the new Society to hold its first annual meeting in the fall of 1928 at the University of Chicago. It was further voted to extend Charter Membership until that time, and the Society Council was authorized to invite additional men at its discretion.

On Nov. 23, 1928, the First Annual Meeting of the Central Society for Clinical Research was held at the Albert Merritt Billings Hospital in Chicago when twenty-two scientific papers were presented.

The further growth and activities of the Central Society for Clinical Research are a matter of record in the Minutes of the meetings, so no further details need be recorded here. A list of the Charter Members is also on record.

It may be of interest, however, to note various trends and changes which have taken place during the twenty-five years since the Society was organized. Ammendments to the Constitution were under discussion nearly continuously, particularly regarding types of membership and qualifications for membership, until 1936, when the present Constitution was adopted. No formal by-laws were ever adopted, but a list of the many "Council Rulings" has served such a purpose. The relationship of the Society to other organizations was under discussion until 1930, when it was finally resolved that the Central Society for Clinical Research be an independent organization, and pay current rates for publication of its abstracts.

The abstracts of papers presented at the Annual Meetings were first published in the American Journal of Clinical Investigation, and then for a number of years in the Journal of the American Medical Association. In 1944 the Society adopted the Journal of Laboratory and Clinical Medicine as its official publication, and since then all abstracts and many full manuscripts have been published in that Journal. The Editor and Editorial Board of the Journal of Laboratory and Clinical Medicine are appointed by the Society.

In the early years, dinner meetings of the entire Society were held after the Scientific sessions. Guest speakers were secured in 1929 and 1930; in 1931 Dr. James B. Herrick read a paper, "Why I Read Chaucer at Seventy," and the 1932 dinner meeting was a memorial meeting in memory of Dr. Billings. In 1933 Dr. Middleton presented the Society with a walnut gavel made from Dr. Billings' homestead. Since 1932 there have been no official dinner meetings.

Annual scientific meetings have been held each fall, even through the war years, and since 1933 meetings have been extended over two days.

A Council Fund was established in 1931 by the generosity of Dr. Billings and Dr. Rowntree; since then the Fund has been augmented by many contributions from emeritus members. The purpose of this Fund is to aid in bearing the expense of attending the meetings by the younger men who are financially unable to attend without this help.

The Society was of some aid in the war effort by submitting to the Division of Medical Science of the National Defense Council a classified list of its members regarding their special qualifications for defense work.

Acknowledgment is made to the Central Interurban Clinical Club and to the Central Clinical Research Club for permission to review the minutes of their meetings.

TEACHERS OF MEDICINE

W. Barry Wood, Jr., M.D.
St. Louis, Mo.

TODAY we celebrate the twenty-fifth anniversary of the Central Society. The story of its genesis has been clearly set forth in print by the first secretary, Dr. Lawrence Thompson. To him and to the other founders, who guided the Society's early destiny, we are deeply indebted.

The chief objective of the Central Society has been to stimulate clinical research. During the past twenty-five years, it has continued to grow in stature, until in 1952 it ranks among leading medical societies of the country. Into its membership has come a steady stream of promising investigators. Their research endeavors in the laboratory and clinic have contributed significantly to the advancement of medicine. But besides being investigators, most of the members of the Central Society have also been clinical teachers. As a group they have exerted a profound influence upon medical education in the Middle West. For this reason, and because medical education is today under fire from a number of directions, I should like to devote my few remarks, not to clinical investigation, but to the teaching of medicine.

The task of the teacher has, during the lifetime of the Central Society, been drastically changed by the progress of science. To illustrate, I should like to cite a single example. One year before the Society was founded, the W. B. Saunders Company published the first edition of Cecil's *Textbook of Medicine*. The section on anemia in this first edition admirably summarizes the subject as taught to students in 1927. But in contrast to the 1951 edition, the presentation contains no reference to any of the following topics: erythroblastosis fetalis; Rh antibodies; abnormal hemoglobins of sickle-cell disease; aplastic crises of congenital hemolytic icterus; normal survival of red blood cells; thalassemia major and minor; paroxysmal nocturnal hemoglobinuria; Coombs' test; extrinsic and intrinsic factors; vitamin B_{12} therapy; pteroylglutamic acid; bone marrow aspiration; absorption and excretion of iron; and pathogenesis of anemia in sprue.

Though not necessarily complete, this list is long enough to indicate that the factual information available concerning anemia has substantially increased since 1927. And all of these new facts must be taught to medical students! Innumerable similar examples could be cited to illustrate the growth of knowledge which has swelled the medical curriculum to the bursting point and has immeasurably increased the complexities of teaching.

As the task of the teacher has grown more difficult, his critics have become more vociferous. Today he is accused of being so absorbed in the scientific

From the Department of Internal Medicine, Washington University School of Medicine.
Presidential address, Twenty-fifth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 7 and 8, 1952.

advances of his subject that he has lost sight of the very objective of his calling—the training of physicians. He is said to have forgotten the art of bedside diagnosis, to have become dependent upon intricate and expensive laboratory tests and to have lost interest in the training of general practitioners. He is censured for ignoring the "patient as a whole" and for slighting the social and psychologic aspects of disease.

Because of these alleged deficiencies certain medical educators, materially aided by a number of foundations, have recently launched a vigorous campaign to reform medical education. They have recommended the introduction of home care programs and undergraduate preceptorships in general practice in order to provide more instruction in the practicalities of medicine. They have suggested the teaching of social sciences in medical school. They have called for more instruction in psychiatry in order to develop in medical students a deeper understanding of the whole person.

Let us examine for a moment these criticisms and suggested remedies.

As to the training of general practitioners, it cannot be denied that there is a need for versatile doctors in isolated communities. Although improvements in transportation have brought rural areas far closer in time to the nearest medical centers, there are still many regions of the country where the needs of the population require the services of general practitioners. With those who recommend more appropriate *postgraduate* training for general practice I have no quarrel. A limited number of internships and residencies designed specifically to prepare physicians to serve in rural communities seems justified. But to introduce such training into the undergraduate curriculum is an entirely different matter.

My disagreement with those who advocate the teaching of general practice in medical schools does not relate to the *need* for general practitioners, but rather to the suggestion that general practice should be taught at all to undergraduates. Surely no one will deny that the unprecedented progress in medicine during the past fifty years has come primarily from the advances of medical science translated into practice through medical education. The volume of basic knowledge which must be mastered by the medical students of today is far greater than ever before. And yet, we are now urged to teach students less about the science of medicine and more about the practical aspects of home care and office practice. Why, may I ask, should the undergraduate curriculum be any different for a general practitioner than for a neurosurgeon? Do they not both have to know a maximum of medical science? Can they, for example, give proper care to their anemic patients if they know nothing of Rh antibodies or the basic principles of iron metabolism?

Many who are not directly involved in the day-to-day teaching of medical students quickly forget that the most effective instruction in medicine is that which involves basic principles rather than practical facts. "For it is," to quote Barzun,[1] "the oldest fallacy about schooling to suppose that it can train a man for 'practical life.' Inevitably while the plan of study is being taught, 'practical life' has moved on." No thoughtful physician needs to be reminded

that what was practical yesterday may be obsolete tomorrow. In contrast, basic principles in medicine are relatively stable. New ones are added from time to time and old ones are occasionally discarded, but the turnover is comparatively slow. Only knowledge based upon principles is sufficiently enduring to aid the modern doctor in his endless task of keeping pace with medicine. In short, general practice should have no more place in the undergraduate curriculum than should the practice of internal medicine or any other kind of practice. Changes in the medical curriculum, which sacrifice training in basic science, either preclinical or clinical, for practical experience, will inevitably lead to inferior medical care.

The real objective of undergraduate instruction is ably stated in the following quotation from a lecture recently published in the *British Medical Journal*.[2] "The student can scarcely be expected to realize what some of his teachers may forget, that when he qualifies it is not so much what he knows that matters as how well fitted he is to learn by his subsequent experience, and how well equipped he is to sift and assimilate the new diagnostic and therapeutic advances which will certainly come during his practicing lifetime. It may once have been true that the practice of medicine did not greatly change between qualification and retirement, but nothing could be further from the truth now. The student who qualified twenty years ago has since had to learn a great deal to remain even tolerably efficient. Of course there are aids to this necessary postgraduate learning, but the doctor must have a fairly well-developed capacity for critical thought and judgment. It is this capacity to examine critically new ideas, to select those worth while, and to incorporate them in the body of his knowledge which alone justifies the claim of the doctor to be a member of one of the learned professions."

To come to the second major criticism of present-day medical education, let us consider briefly the proper place of psychosomatic medicine in the undergraduate curriculum. Here I am quite willing to admit that as clinical teachers we frequently fail to lay adequate stress upon the psychological aspects of illness. Our shortcomings in this respect seem to stem primarily from two sources.

First, many of us have fallen far behind in our understanding of psychosomatic medicine. Too much in this field has seemed to us in the past to have been based upon flimsy evidence, or no evidence at all. As a result, we have tended to refer all problems of the psyche to the psychiatrist, or have merely ignored them altogether. Such a position is no longer tenable. Research from the clinics of Wolff and Romano, for example, have been yielding for at least a decade concepts based upon as clear scientific evidence as those of any other branch of clinical medicine. To deny the importance of psychological factors in such diseases as bronchial asthma, ulcerative colitis, peptic ulcer, hypertension, and urticaria, to mention but a few, is to play the ostrich and bury our heads in the familiar sands of purely somatic reasoning.

But the solution to the problem does not lie in the training of more psychiatrists nor in the establishment of new departments of psychosomatic medicine. The subject matter belongs in internal medicine, pediatrics, surgery,

and obstetrics, and it must be so taught to medical students. Foundations supporting developments in this field have erred in insisting upon the establishment of separate "departments" or "divisions" of psychosomatic medicine. The inevitable degree of autonomy thus created makes for easier bookkeeping, but at the same time tends to defeat the main objective of the program. How much more would be accomplished if psychosomatic experts were "planted" in the already existing departments, experts who would function as clinicians and not merely as consulting "psychosomaticists." We must have such colleagues in our very midst, as we now have experts in cardiology, metabolism, hematology, and the other sub-specialties of medicine, if we are ever to convince undergraduate students that this important field belongs in everyday clinical practice.

The second factor which makes the teaching of psychosomatic medicine so difficult is that of time. Accurate appraisal of the psychological aspects of a given patient's illness depends primarily upon the anamnesis, which, when properly taken, is to the physician by far the most time-consuming part of the examination. It must include adequate personal and social histories, and these require even more of the doctor's time than the conventional system review and present illness. If he is to treat the "patient as a whole," the physician must know "the patient as a whole." To do so, he must devote to each case not minutes but hours. As long as doctors are chronically rushed, the majority will fail to practice "comprehensive medicine."

Does it follow that "comprehensive medicine" should not be taught to undergraduates? Certainly one of the main functions of medical schools is to foster change that will raise the standards of medical care. As teachers we can do much to encourage the physicians of the future to adopt a more comprehensive approach to the study and treatment of disease. In so doing, however, we, too, will be limited by time. To teach comprehensively, we shall have to see fewer patients with our students on ward rounds, and assign to them fewer cases in the clinic. We shall have to sacrifice volume for completeness. Such a trend has already been forced upon us by the technical advances of laboratory medicine. Since most major errors of clinical practice result from sins of omission, and since physicians all too readily learn to cut corners after finishing their formal training, I am convinced that a sacrifice of volume for completeness is pedagogically sound.

Finally, I should like to refer to the most serious problem of all in medical education; namely, that created by increasing specialization. An inevitable result of advancing knowledge, specialization today is exerting a powerful disrupting force upon all of higher education. To meet the problem in medicine particularly will require much wisdom, for outside pressures which exaggerate the difficulty are constantly increasing. Special groups are clamoring for more time in the curriculum. Funds are being made available through Congress for substantial support of special areas. Medical specialists through both local and nationwide drives are obtaining vast amounts of money from the public to further special projects. Even foundations, attempting to create

better balance in medical education, are providing temporary support for what they consider to be neglected specialties.

I have referred advisedly to these forces as "outside pressures," for those who control their direction are, for the most part, men whose daily work has divorced them from the undergraduate teaching of medicine. The sensory end organ of the medical school most responsive to these pressures is "the administration," which likewise is often so burdened with other responsibilities as to have no time for teaching. This combination, which threatens to control the policies of medical schools, bypasses the teachers of medicine. It has already shown itself to be much too willing to mortgage the future, in order merely to meet the public pressures of the moment. If medical education is to continue to flourish, the *educational* policies of medical schools must remain in the hands of faculties and not of professional administrators. Only teachers devoting their lives to the core of learning are close enough to the complex problems of their profession to plan wisely the education of the future.

Whether or not society will continue to allow teachers of medicine to guide the destinies of medical schools will depend primarily upon our performance as educators. Dedicated to "the maintenance of balance between the advancement of knowledge and its consolidation,"[3] we must somehow find means of consolidating the deluge of new facts to be taught to our students. We must neutralize the centrifugal force of specialization. Under no circumstances must we permit the availability of project funds to determine our decisions of educational policy. Those of us in clinical practice must donate from busy days not only the necessary hours for teaching, but also enough additional time to keep sufficiently abreast of medical progress to remain properly qualified as teachers. Those of us on "full time" must refrain from using our university appointments solely "to boil the pot of individual research." Outside duties which take us away from our institutions must not be allowed to cut too deeply into undergraduate instruction. Those of us who by preference would limit our efforts solely to postgraduate teaching must also participate in the undergraduate program. In fact, all of us as faculty members must continually be reminded of our obligation to teach medical students. For upon the qualifications of those whom we graduate will depend the future of American medicine.

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THE RELATION OF LIFE STRESS AND EMOTIONS TO HUMAN SEBUM SECRETION AND TO THE MECHANISM OF ACNE VULGARIS

THOMAS H. LORENZ, M.D.,* DAVID T. GRAHAM, M.D.,** AND STEWART WOLF, M.D.***

NEW YORK, N. Y.

INTRODUCTION

ACNE vulgaris is generally considered to be a disorder of the sebaceous glands of the human face, upper chest, and back. The primary lesion of acne is the comedo[14] or "blackhead," which is a plug in an oil gland duct of the skin composed of various combinations of sebum and keratin.

Although the exact etiology of this disorder has remained unknown, a multitude of factors have come to be regarded as predisposing or contributing forces in the pathogenesis and course of acne vulgaris.[15] Among them, emotional stress has been considered to be important.

As a point of departure for the present study, it was postulated that, if life stress and emotional reactions were pertinent to the acne mechanism, it was probably because of associated quantitative or qualitative alterations in sebum secretion. Accordingly, when a new and simple method for the quantitative estimation of surface sebum became available,[9] it was utilized to investigate the proposed problem of the possible relationship between sebum secretion and emotions.

SUBJECTS

Thirty patients obtained at random from various clinics of the New York Hospital where they sought treatment for their chief complaint of "acne" were studied in detail. Except for the presence of acne vulgaris, these patients were unselected. Specifically, they were not chosen because they were originally considered to be "nervous" or to have emotional problems complicating their skin disorder. (It was recognized that the patient's willingness to come to the clinic introduced a possible selective factor; yet this defect is shared by most, if not all, so-called groups or series of patients involved in outpatient clinical investigations.) Twenty-two were females and eight were males.

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*Present address: Department of Medicine, University of Wisconsin Medical School, Madison, Wis.

**Present address: Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

***Present address: Department of Medicine, University of Oklahoma School of Medicine, Oklahoma City, Okla.

They ranged in age from 15 to 31 years; the average age was 22 years. Each of these patients was seen for a total of at least two hours, and some were seen for as many as twenty hours. All of the patients had careful physical examinations with appropriate laboratory studies. In addition, most of them had basal metabolic rate and serum cholesterol determinations. In only two cases was the basal metabolic rate below −10. In one of them the cholesterol was 185 mg. per cent and in the other 200 mg. per cent.

Control observations were made on 10 nonacne patients from the medical clinic. The only criteria used in the selection of the control group were that they fell within the age range of 15 to 31 years, and that they did not have acne vulgaris. The average age of this group was 23 years. Seven were females and three were males. The primary diagnosis in five cases was migraine, while in the other five the diagnoses were: irritable colon, asthma, vasomotor rhinitis, neurodermatitis, and anxiety tension state.

During outpatient visits a relatively complete life history was obtained from the patients. In addition, each was carefully evaluated from a psychological standpoint, with special attention accorded his emotional status and level of life adjustment, both past and present. Long-term observations performed on the acne patients included: (1) daily sebum determinations for thirty days, and (2) "diary" notes which included data on events of day-to-day life, emotional and other reactions to them, and day-to-day "pustule count."

The significance of the temporal coincidence of apparently stressful events and changes in "pustule count" was verified in short-term experimental studies in which topics of supposed conflict were discussed while surface sebum was being measured. These observations were controlled by measurements made during a discussion of topics which were not stressful for those particular individuals.

METHOD OF SEBUM ESTIMATION*

Collection of Sebum Specimens.—As described in detail elsewhere,[11] a convenient and reliable means for collecting surface sebum specimens was devised in which the ground end of a clean glass rod was applied to a properly prepared skin surface under standardized conditions of pressure and time. A sketch of the apparatus employed for this purpose is shown in Fig. 1. It consisted essentially of a piston shaft, one end of which was equipped with a metal sleeve for holding the glass rod and the other end of which was secured to a coiled spring fitted into the hollow handle of the device. Pressure of application was measured by the extent to which the handle descended the piston shaft and the scale etched thereon. A pressure of 400 Gm. for five seconds was used in all collections. The procedure was not at all uncomfortable.

*The material actually collected by this method is surface lipid, which consists not only of the fatty material extruded on to the skin surface from the sebaceous glands (sebum), but also that from the horny layer of the epidermis and possibly from the sweat glands. There is, however, evidence that the sweat glands do not contribute significantly,[11] and it seems reasonable to assume that the amount coming from the epidermis is relatively small and constant. We have therefore followed the practice of other investigators in referring to the surface lipid as "sebum," and in considering changes in the surface lipid as reflections of changes in sebaceous gland function.

At an appropriate time prior to starting a sebum collection, the skin of the face was cleansed with cotton pledgets immersed in ether. Thus the quantity of each sebum specimen collected depended upon the amount of sebum reappearing on the skin surface.

The site of sebum collection used in this investigation was the "angle of the nose." The selection of this site was based on the results of preliminary studies[11, 18] which demonstrated that it was the most sebum-rich area of the face, and that alterations in facial sebum secretion were not localized and selective but usually occurred together in the four areas examined (forehead, cheek, chin, and nose). The point of collection at the "angle of the nose" was as nearly as possible identical in all collections from the same and different subjects.

Determination of Sebum Specimens.—The method of Berry[1] and Jones[9] was utilized for the quantitative determination of sebum specimens in this investigation. The essential principle involved in this method is that, when fatty material is placed in contact with a monomolecular oil layer, it will spread as a film against the oil. The area of spread of such a film is a sensitive and accurate index of the amount of fat delivered to the oil surface.[9]

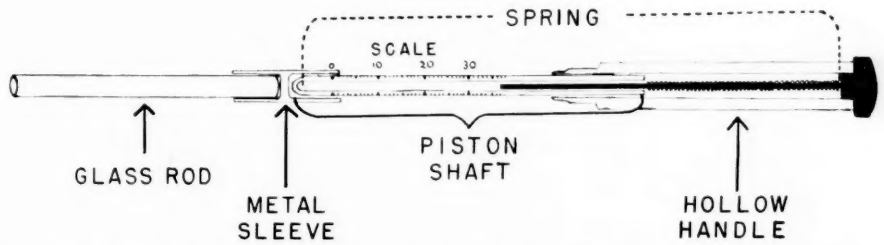

Fig. 1.—Sketch of modified algesiometer adapted for use as a rod holder and providing for standardized pressure of glass rods on skin surface.

Thus, in the present study, the sebum-tipped rod was touched to the surface of such a monomolecular layer of oil, and the resultant area of spread was taken as a measure of the amount of the sebum specimen.

Planimetric measurement of the outline of the sebum film permitted expression of the quantity of the specimen in square inches.

OBSERVATIONS

Characteristic Affective Reaction, Attitude, and Gross Fluctuations in Facial Seborrhea.—A comprehensive report covering these will be published elsewhere.[12] In essence a characteristic emotional pattern was identified in the acne patients, which consisted of episodes of intense "anger" promptly followed by more prolonged periods of "remorse" (guilt and depression). Furthermore, this characteristic affective reaction occurred most commonly when the patients found themselves in conflict with some figure of authority, usually a parent. In these circumstances they believed their highly valued, but unrealistic, sense of independence and individuality was being threatened or abused. This provoked them to "anger" but only briefly as a rule, for, whether expressed or not, aggressive and hostile feelings were viewed as "bad." Thus, guilt and

depression intervened, and persisted for a few hours or even a few days, in the wake of their "righteous wrath."

The predominant attitude statement elicited from these patients, which was associated with both phases of their characteristic emotional pattern, was: "I wish they would let me alone and quit picking on me." There was, however, a difference between what these patients wanted to do about the situations they found themselves in when they were angry as compared with the phase when they were remorseful. In the former, they threatened or wished to take aggressive action if they were not left in peace, while in the latter they felt it was futile even to consider taking action themselves, leaving the responsibility for the solution of their problems to those about them.

The majority of patients regarded their facial skin as being grossly "more oily than the average person's"; yet most of them observed that this was not *always* so. At times their faces "were oily and at other times dry—for no reason at all." By the latter statement the patients meant that decrease in facial skin oil was not due to any change in their washing and cosmetic habits, diet, activity, etc.—"it just happened." Only rarely did patients initially volunteer the information that such fluctuations in facial sebum secretion might be related to their feeling states and life stress.

LONG-TERM OBERVATIONS

Daily Sebum Determinations.—

Procedure: In order to obtain data on the day-to-day fluctuations in surface sebum output in patients with acne vulgaris, daily sebum collections were made under standard conditions (from the "angle of the nose," approximately one hour after washing and at the same time every day) from six acne subjects for thirty days. Six nonacne subjects were followed in a similar manner.[11]

Results: As illustrated in Fig. 2, a relatively marked fluctuation in facial sebum output was found to occur from day to day in acne patients; however, this was not essentially dissimilar to that found in nonacne subjects. Although on some of the days when there was increased sebum output the acne patients were angry and on other days when their facial sebum secretion was decreased they were depressed, there were periods when the range of sebum values was equally marked, but no mood deviations could be discerned. This corresponds completely to the findings in nonacne subjects and is consistent with the belief that no *single* factor is solely responsible for such *day-to-day* alterations in facial sebum output.[11]

Diary and Daily "Pustule Count."—

Procedure: Twenty-two of the acne patients were instructed to keep a diary which included a record of their daily experiences, what these meant to them, and how they reacted to them, as well as a record of their total facial "pustule count" from day to day. (The latter was selected as a rough indicator of the state of their acne for a number of reasons: [1] it was impossible for the patient to count his comedones, [2] it was impractical for him to count the number of papular lesions on his face, but [3] it was comparatively easy for him to keep count of the number of "whiteheads" since these were obvious, well-defined lesions and usually few enough in number so as not to be confusing to the patient.)

Results: These data will be presented in detail elsewhere,[12] but essentially it was demonstrated that a close correlation existed in this group of patients between their basic emotional reactions of "anger" and "remorse" and exacerbations in their acne pustules.

Figs. 3 and 4 graphically portray an example of the day-to-day mood variations and fluctuations in facial pustules which occurred over a six-week period of time in one of the patients in this series.

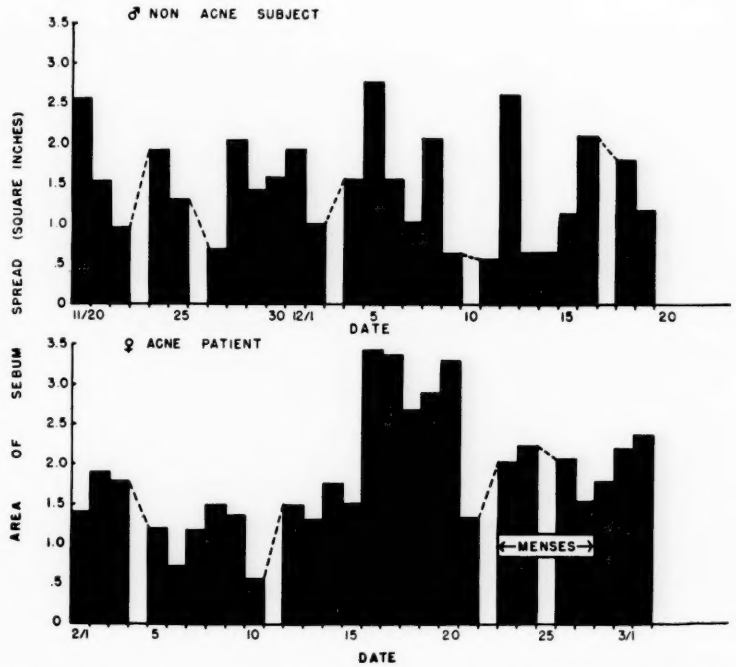

Fig. 2.—Daily sebum determinations over a one-month period in a healthy subject and in an acne patient. Note relatively marked fluctuation in facial sebum output from day to day in each instance.

SHORT-TERM EXPERIMENTAL OBSERVATIONS

In 25 of the 30 patients short-term experimental observations of sebum secretion were made periodically during interviews which were conducted in such a way as to be stressful (through discussion of emotionally charged material as well as by manipulating the doctor-patient relationship) or non-stressful (by avoiding the aforementioned topics and maneuvers) for these patients. Insofar as possible, these experiments were undertaken during periods in which the patients were at least initially relatively neutral from an emotional standpoint.

It was found that, when the physician's attitude indicated unhurried sympathetic understanding, a feeling of relaxation and tranquility was induced in these patients. On the other hand, an abrupt change in the physician's attitude in which he took on the role of a critical, unsympathetic, obstinate and

inquiring figure in authority proved very stressful for this group of patients. The majority of acne patients were provoked by such a situation to intense feelings of irritation and anger, although in some individuals feelings of guilt and depression predominated. Either type of response, however, could be dissipated quite regularly if the physician discarded his unpleasant and provocative behavior and returned completely to the role of a pleasant, understanding, and sympathetic therapist.

During the process of such an interview, particular note was made of alterations in the patient's appearance, speech, and behavior, which often were valuable concurrent guides to the success of the physician's intentions. In addition, at the termination of the experimental period the patients were asked to appraise their own feelings, reactions, and attitudes during the different portions of the interview. This precautionary measure was necessary in getting an estimate of the physician's success or failure in producing a stressful or nonstressful situation, since it was not always possible to control satisfactorily the emotional tone of an interview.

It should be noted that this method entailed the conscious effort of the physician to follow a *prescribed pattern* in the conduct of each experimental study. This was designed to subject the patient to a particular type of stress for a limited and well-defined period of time and to avoid such stress at all other times.

During each portion of these experiments periodic sebum collections were made.

Thus, on the completion of this type of experiment, any results, in terms of alteration in the output of facial sebum, could be viewed properly in the light of the stress, or lack of it, to which the patient had been subjected during the interview.

The experiments were of three general types:

Nonstress Experiments—Acne Patients.—

Procedure: These interviews were conducted for one hour. Throughout this period ("control" one-half hour and "test" one-half hour) only neutral topics were discussed, and the physician maintained a friendly, sympathetic, and helpful attitude toward the pa-

TABLE I. SUMMARY OF RESULTS OF ONE-HOUR EXPERIMENTS IN WHICH MEASUREMENTS OF SEBUM SECRETION WERE OBTAINED DURING INTERVIEWS MADE STRESSFUL OR NONSTRESSFUL FOR ACNE AND NONACNE PATIENTS

TYPE OF EXPERIMENT	NUMBER OF OBSERVATIONS	MEAN VALUE SEBUM SPREAD DURING CONTROL PERIOD (½ HR.)	MEAN PER CENT CHANGE DURING TEST PERIOD (½ HR.)	RANGE PER CENT CHANGE
Acne: stress				
"Anger"	29	0.67 sq. in.	+168	+51 to +440
"Remorse"	5*	1.84 sq. in.	− 61	−46 to − 76
Acne: nonstress	38	0.73 sq. in.	+ 3	−29 to + 34
Nonacne: stress	10	0.95 sq. in.	+ 0.7	−14 to + 20

*In 2 cases sebum collections were made at fifteen-minute, instead of thirty-minute, intervals.

tient. If emotionally charged material inadvertently entered the discussion, the topic of conversation was changed to nonstressful subjects. Sebum collections were usually made thirty minutes after washing, midway through the interview, and at the end of the experiment. On some occasions, four fifteen-minute sebum collections were made instead of two thirty-minute collections.

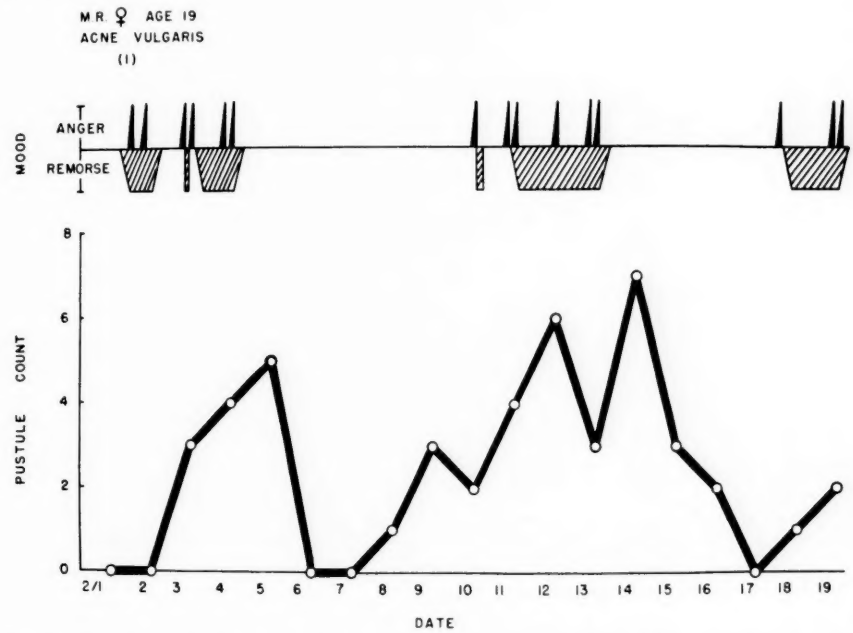

Fig. 3.—Correlation between phasic mood alterations and number of facial pustules in a patient with acne vulgaris during a six-week period.

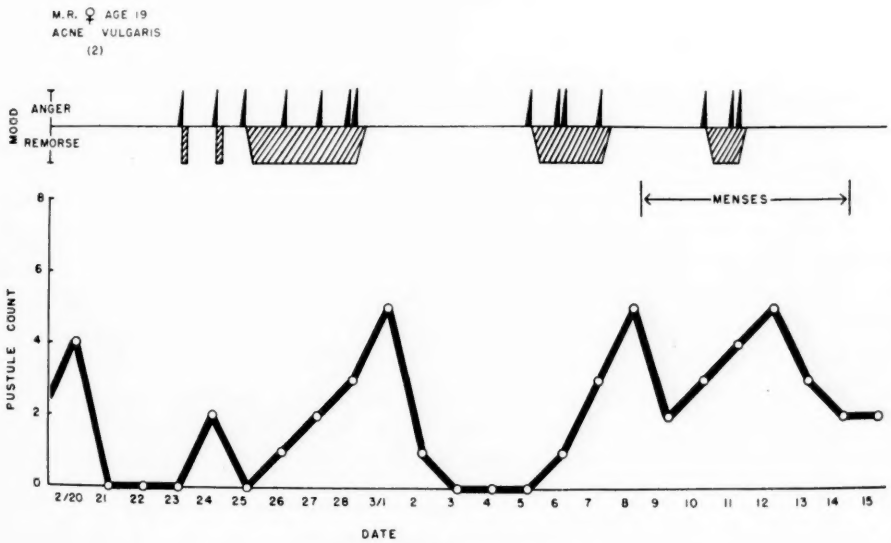

Fig. 4.—Continuation of Fig. 3.

Results: In the 38 one-hour nonstress experiments performed on 25 acne patients, the mean "control" sebum value (one-half hour) was 0.73 square inch. In no instance did a significant change from the "control" facial sebum output occur during the nonstressful "test" half hour in these experiments. The mean change in facial sebum secretion obtained during this "test" period was plus 3 per cent, with a range of minus 29 per cent to plus 34 per cent (see Table I).

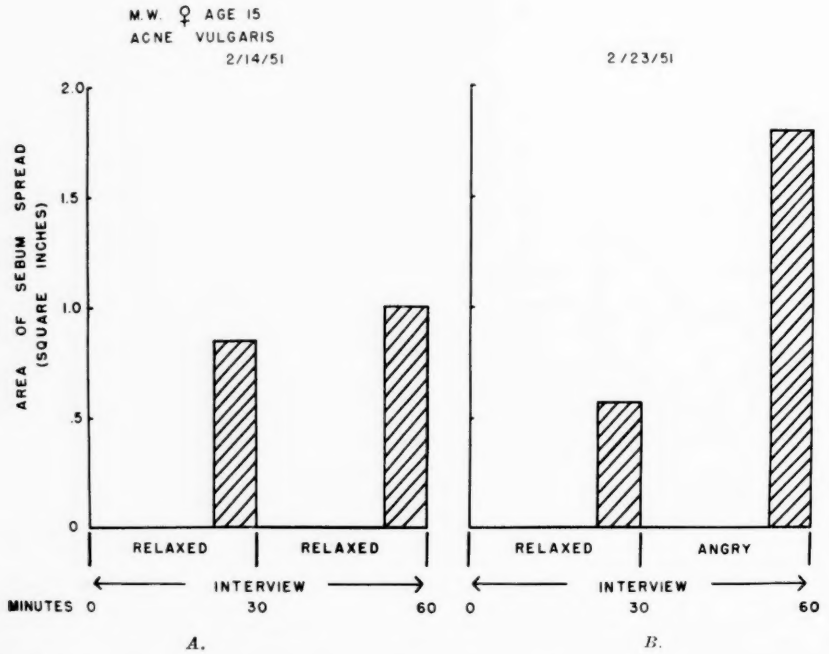

Fig. 5.—Results of one-hour nonstress (*A*) and stress (*B*) experiments on youngest acne patient in series. Note essentially unaltered facial sebum output during one-hour nonstress experiment (*A*), as compared to increased output during stress experiment—in which "anger" was induced (*B*).

Figs. 5, *A*, 6, *A*, and 7, *A* illustrate the absence of significant change in facial sebum output during one-hour nonstress experiments as compared to that found in one-hour stress experiments on the same acne patients ("M. W." was the youngest acne patient in the group and "J. S." was the oldest. Since both of these patients were females, "A. G." was included as representative of the male acne patients).

These results are similar to previously reported findings in nonacne subjects which showed that during tranquil periods (for as long as ninety minutes) facial sebum output could be maintained at practically a uniform and stable level.[11]

Stress Experiments—Acne Patients.—

Procedure: Here the procedure was identical with that of the nonstress experiments except that the second ("test") half hour was made stressful for the patient, inducing in him

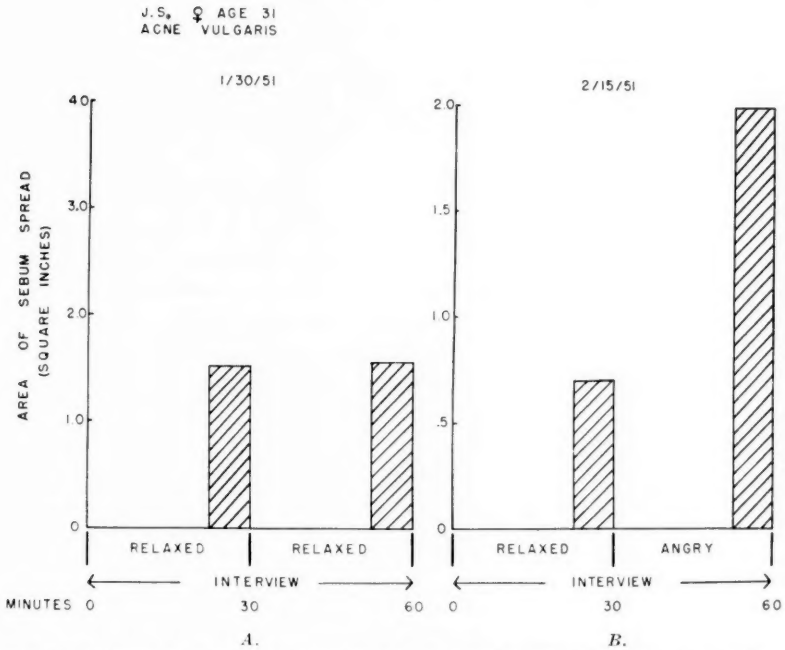

Fig. 6.—Results of one-hour nonstress (*A*) and stress (*B*) experiments on oldest acne patient in series. Note essentially unaltered facial sebum output during one-hour nonstress experiment (*A*), as compared to increased output during stress experiment—in which "anger" was induced (*B*).

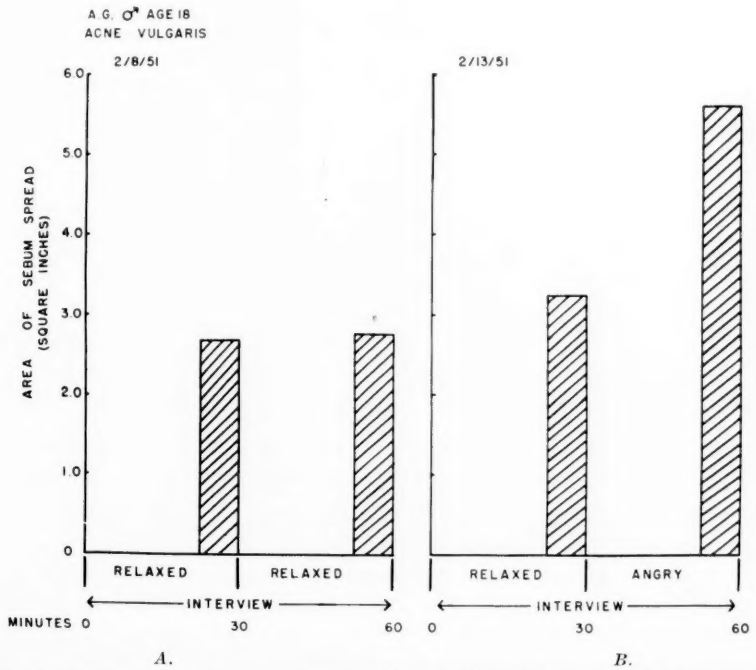

Fig. 7.—Results of one-hour nonstress (*A*) and stress (*B*) experiments on representative male acne patient in series. Note essentially unaltered facial sebum output during one-hour nonstress experiment (*A*), as compared to increased output during stress experiment—in which "anger" was induced (*B*).

a response of either "anger" or "remorse" (in association with the described attitudes) on the part of the patient.

Some of the stress experiments were prolonged to ninety minutes by adding an additional half hour which was devoted to strongly reassuring the patient, and re-establishing within him a feeling of security and relaxation. In these cases, fifteen-minute sebum collections were made throughout, to a total of six.

Results: In 29 one-hour stress experiments, during which "anger" was induced in 20 acne patients, the mean "control" sebum value (one-half hour) was 0.67 square inch. In *every* instance there was increased facial sebum output during the "test" portion of these interviews, i.e., when the patients were reacting with "anger." A mean rise of 168 per cent, with a range from plus 51 per cent to plus 440 per cent, occurred during this stressful period (see Table I). Figs. 5, *B*, 6, *B*, and 7, *B* illustrate the typical abrupt increase in facial sebum output which occurred in these one-hour stress experiments on acne patients in whom "anger" was induced.

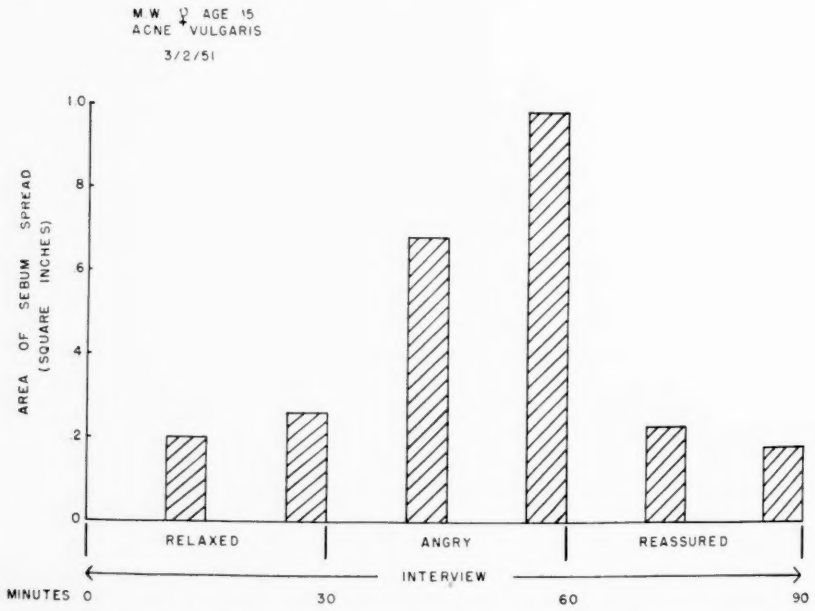

Fig. 8.—Results of ninety-minute stress experiment on youngest acne patient in series. Note increased facial sebum output during stress period, in which "anger" was induced, and decreased output during reassurance.

In the 12 ninety-minute stress experiments, during which "anger" was induced in the "test" half hour in 12 acne patients, the mean "control" sebum value was 0.51 sq. in., the mean sebum value during the "test" period was 1.15 sq. in., and in the final period devoted to reassurance it was 0.50 square inch. Again, in *every* instance there was a rise in sebum output, as compared to the "control" value, during the stressful portion of these experiments. The mean rise was 173 per cent with a range from plus 64 per cent to plus 445 per cent. Uniformly, there was a decrease in sebum output, as compared with the stress level, during reassurance. The mean fall with reassurance was 54 per

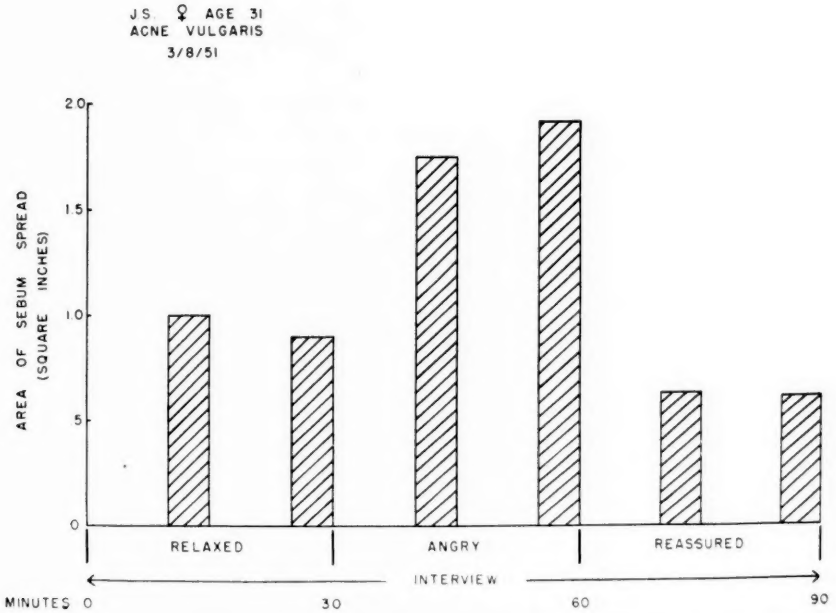

Fig. 9.—Results of ninety-minute stress experiment on oldest acne patient in series. Note increased facial sebum output during stress period, in which "anger" was induced, and decreased output during reassurance.

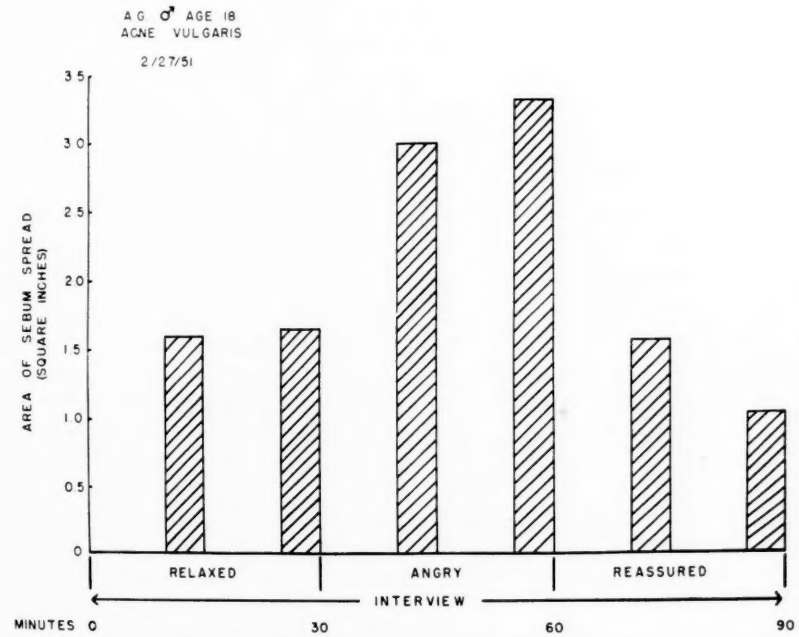

Fig. 10.—Results of ninety-minute stress experiment on representative male acne patient in series. Note increased facial sebum output during stress period, in which "anger" was induced, and decreased output during reassurance.

TABLE II. SUMMARY OF RESULTS OF NINETY-MINUTE STRESS EXPERIMENTS PERFORMED ON 12 ACNE PATIENTS IN WHICH 2 FIFTEEN-MINUTE SEBUM MEASUREMENTS WERE OBTAINED DURING EACH OF 3 ONE-HALF-HOUR PERIODS: "CONTROL," "STRESS," AND "REASSURANCE"

PATIENT	AGE	SEX	AV. VALUE SEBUM SPREAD DURING CONTROL PERIOD	AV. VALUE SEBUM SPREAD DURING STRESS PERIOD	PER CENT CHANGE DURING STRESS "ANGER"	AV. VALUE SEBUM SPREAD DURING REASSURANCE PERIOD	PER CENT CHANGE DURING REASSURANCE PERIOD
D. M.	20	M	0.59 sq. in.	1.16 sq. in.	+ 96	0.33 sq. in.	−71
A. G.	18	M	1.63 sq. in.	3.17 sq. in.	+ 94	1.31 sq. in.	−58
H. G.	21	M	0.56 sq. in.	0.92 sq. in.	+ 64	0.68 sq. in.	−26
L. H.	22	M	0.12 sq. in.	0.38 sq. in.	+216	0.09 sq. in.	−76
E. G.	19	M	0.12 sq. in.	0.25 sq. in.	+108	0.14 sq. in.	−44
M. R.	19	F	0.11 sq. in.	0.60 sq. in.	+445	0.33 sq. in.	−45
A. F.	19	F	0.19 sq. in.	0.82 sq. in.	+331	0.35 sq. in.	−57
M. B.	19	F	0.69 sq. in.	1.64 sq. in.	+137	0.70 sq. in.	−57
R. S.	25	F	0.47 sq. in.	1.01 sq. in.	+114	0.43 sq. in.	−57
M. W.	15	F	0.23 sq. in.	0.83 sq. in.	+260	0.21 sq. in.	−74
J. S.	31	F	0.95 sq. in.	1.83 sq. in.	+ 92	0.63 sq. in.	−65
B. Q.	25	F	0.54 sq. in.	1.24 sq. in.	+129	0.91 sq. in.	−26

Note consistent increase in sebum output with stress, inducing "anger," and consistent fall with reassurance.

cent of the stress value, with a range of minus 26 per cent to minus 76 per cent (see Table II). Figs. 8, 9, and 10 demonstrate the typical increase in facial sebum output found during the stressful portion of these experiments, when the acne patients were provoked to "anger" and the subsequent fall in sebum output during the period of strong reassurance.

In most instances when these acne patients became angry, transient facial flushing was apparent. In addition, evidence of increased psychomotor activity was present with heightened rate of speech, increased muscle tension, and restlessness. None of these was evident when they were remorseful.

It is noteworthy that, although sweating was not grossly evident during these stress experiments, previous studies[11] indicated that the presence of sweat made little or no difference in the sebum readings obtained.

In the five patients in whom feelings of "remorse" were mobilized during the stressful "test" period there was in each case a decrease in facial sebum output as compared to the "control" value. The mean fall was 61 per cent, with a range from minus 46 per cent to minus 76 per cent (see Table I). In two of these cases, four fifteen-minute sebum collections were made during the interview, one during an initial fifteen-minute control period, two during a thirty-minute period of stress, and one at the end of a fifteen-minute period of reassurance. In the other three experiments the time relations were the same as in the "anger" studies. Fig. 11 is representative, and shows the decrease in facial sebum output which occurred in one acne patient during the stressful portion of an interview when feelings of "remorse" were produced. Note the return of the sebum value toward the "control" level during the period of reassurance.

Stress Experiments—Nonacne Patients.—It was important to know whether the changes in facial sebum observed in the acne patients under stress were relevant to their disease or were simply responses which would occur in

any one. Therefore, ten patients who did *not* have acne, but who were known to be responding to stress, were studied.

Procedure: These experiments were conducted exactly like the one-hour stress studies described above, in which two thirty-minute sebum collections were made during the hour. The first (''control'') half hour of these interviews was kept nonstressful for the patient, but the second (''test'') half hour was made stressful.

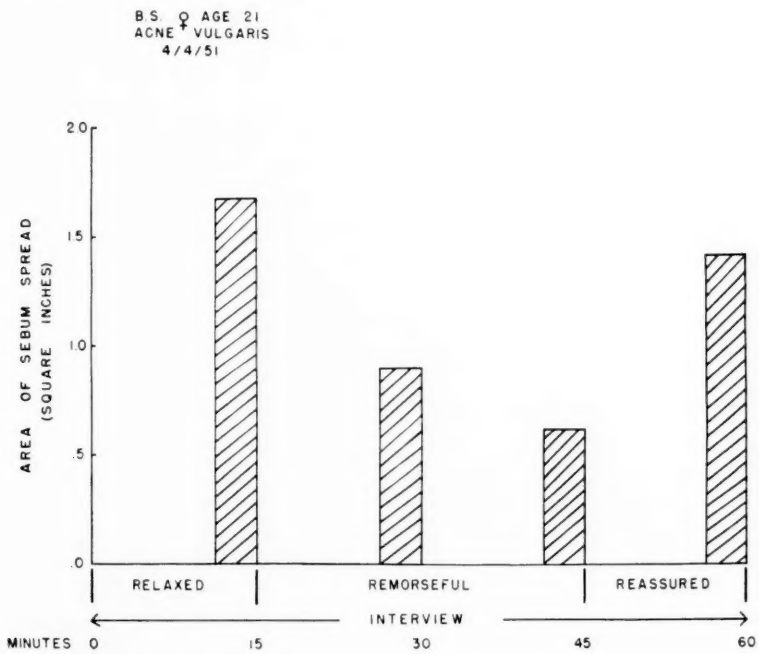

Fig. 11.—Results of one-hour stress experiment on acne patient in whom feelings of "remorse" were mobilized. Note decreased facial sebum output during stress period and return of output toward control level during reassurance.

It should be noted that, in the instance of these nonacne patients, the material discussed and the physician's attitude were selected on the basis of what was appropriate and pertinent to each of these patients and the particular variety of stress disorder he had. The ''stress'' was not necessarily identical in content or meaning to these patients with that to which the acne subjects were exposed. That the nonacne patients in this study were subjected to emotional stress, however, and responded to it in their own manner, was substantiated by observing and questioning them. This will be evident upon examination of the data to be presented.

Results: In the one-hour stress experiments performed on 10 nonacne patients, the mean ''control'' sebum value (one-half hour) was 0.95 square inch. Unlike the acne patients, these patients did *not* show a significant change in facial sebum output when they were subjected and reacted to stress during the ''test'' half hour of these studies. Here, the mean change in facial sebum output during the stressful period was plus 0.7 per cent, with a range of minus 14 per cent to plus 20 per cent (Table I).

That the stress to which these nonacne patients were exposed was effective and meaningful to them was evidenced by the fact that all of them reacted

with mood changes—becoming resentful, angry, frustrated, or depressed—and, in addition, most had observable physiological changes. Each of the migraine patients became flushed when angry, and three of them developed typical vascular headaches before the interview was over. The patient with asthma (and vasomotor rhinitis) became "choked up" during the stressful period. Associated with this there were hyperventilation, coughing, and nasal congestion. Another patient with rhinitis complained of "nasal stuffiness" and obstruction which had not been manifest during the "control" period. The remaining

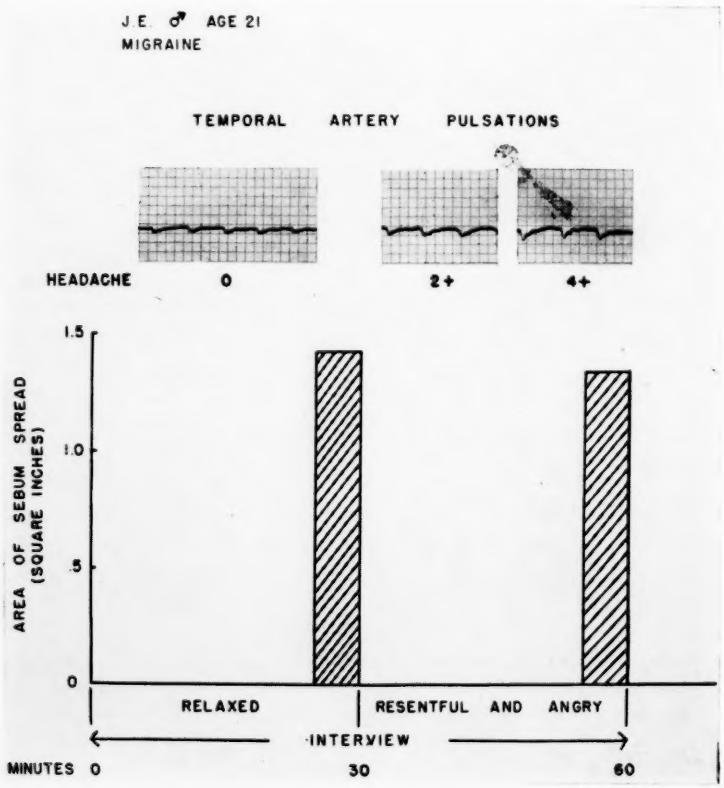

Fig. 12.—Results of one-hour stress experiment on nonacne (migraine) patient. Note essentially unaltered facial sebum output during stress period—in which anger and resentment were provoked and a 4 plus headache occurred in association with altered temporal artery pulsations.

patients showed increased muscular activity, but few other bodily changes, during the stress portion of these experiments.

Fig. 12 illustrates the effect of such a stress experiment upon one migraine patient in whom temporal artery pulsations were recorded throughout the interview. Note the alteration in pulse wave pattern coincident with the appearance of headache during the "test" period, and the absence of any significant change in facial sebum output during the same time.

COMMENT

Statistical measures of significance of differences are not presented. Inspection of the data makes it obvious, in view of the marked differences in results between the various groups and the absence of overlapping, that the probability that the differences are due to chance variations is less than the usually accepted value of 0.01.

DISCUSSION

The results of this study reveal a number of similarities, as well as one (seemingly critical) difference, between acne and nonacne patients in regard to sebum secretion. It has been shown that (1) the "control" level of facial sebum output is practically the same in acne and nonacne patients, (2) during periods of tranquility, when both types of subjects are relaxed, facial sebum secretion is remarkably stable, and (3) there are fairly marked day-to-day (twenty-four-hour) fluctuations in facial sebum output, but the magnitude of these changes is practically identical in acne and nonacne patients. The unique feature of sebaceous gland function found to exist in acne patients is that of *lability,* resulting in prompt fluctuation in facial sebum output under stressful circumstances. Thus, acne patients respond abruptly with either an increased or decreased rate of facial sebum secretion at times of emotional stress, whereas those without acne do not behave in this way. (Although this characteristic conceivably might be related to sebaceous gland hypertrophy in patients with acne, the reasons for such hypertrophy remain to be elucidated.)

The method of quantitative estimation of sebum used in this investigation does not yield data on the absolute amount of surface sebum, yet the finding of similar mean "control" values of facial sebum output in acne and nonacne patients, when they are relaxed, suggests that there is no significant difference in the degree of "facial oiliness" in these two groups. Likewise, the similarity in the range of twenty-four-hour fluctuations in facial sebum output is consistent with this observation. (On the basis of these data, it appears likely that the explanation for the commonly-held impression that acne patients have "more oily" skin than others is that they do—but only periodically, not constantly.)

Although not described in detail, evaluation of the acne patients in this series revealed all of them to have a common pattern of emotional behavior. Each of these patients could be said to have a *labile* affect. This was not a haphazard lability, however, but one which seemed highly characteristic and stereotyped. They responded periodically with feelings of intense "anger" for a brief period of time, promptly followed by a more prolonged feeling of "remorse," when they were "picked on" by those about them and their wish was to be just "let alone." Thus, the daily lives of these acne patients were punctuated by marked swings in affect which had a pattern and meaning that seemed quite specific, if not characteristic, for this group of patients.

Demonstration, in an experimental setting, of the close association between "anger" and seborrhea on the one hand, and "remorse" with decreased

sebum output on the other, revealed that such emotional reactions were intimately related to sebaceous gland activity in these acne patients. (The fact that this phenomenon occurs in association with certain mood states does not mean that *other* factors could not also be associated with such rapid fluctuations in facial sebum secretion in acne patients. However, the pertinence of such "other" factors remains to be proved.)

When a record was kept of these episodic emotional reactions in the acne patients in this series and the number of their facial pustules, over a period of six weeks, it was found that a close correlation existed between these two phenomena. This, then, suggested that such rapid, phasic mood swings were relevant to the clinical state of acne vulgaris in these patients.

As noted above, there is a consensus that acne vulgaris is a disease of the sebaceous glands, and that the primary lesion of this disorder is the comedo. The cause of the comedo, however, is still obscure. Hyperkeratosis of the neck of the sebaceous gland follicle commonly has been observed in acne vulgaris and is considered by "most modern authors" to be the initial step in comedo formation.[16] This belief is not universally held, however. Hamilton, for instance, has stated that the "mere presence (of such epithelial hyperplasia) affords no proof of a primary or even major function in acnegenesis, and the usual blind acceptance of this dogma must be criticized until conclusive evidence is gained."[7] Furthermore, Lynch, in a careful histologic study of early acne lesions, found "no clue as to the cause of the comedo."[13] Increased activity of the sebaceous glands and seborrhea have also been regarded as the primary cause of the comedo.[17] (This, incidentally, might also be responsible for hyperkeratosis, since Hass has demonstrated experimentally that the local irritant effect of lipids may, in itself, produce epithelial hyperplasia in the skin.[8]) The exact mechanism by which such alterations in sebum output produce comedo formation has not been clearly established, however.

On the basis of this investigation, in which it has been demonstrated that patients with acne vulgaris have a unique lability in sebaceous gland activity, it is suggested that abrupt *phasic* alteration in the rate of sebum secretion is an important factor in comedo formation, and therefore in the mechanism of acne vulgaris.

It seems not unreasonable to suppose that during the hyposecretory phase there is in the sebaceous gland relative stasis of sebum, with consequent inspissation and other physicochemical changes which tend toward plug formation. With the next hypersecretory phase, which amounts to a sudden attempt to excrete sebum through a completely or partially "stoppered" duct, the mass of sebum may well become impacted in the duct. Inflammation would be expected to develop around such a plugged gland, and typical papular and pustular lesions of acne would appear. This chain of events would require some time to take place, and it is understandable that a twenty-four- to forty-eight-hour time lag was found to occur between the phasic emotional reactions of the acne patients and changes in their facial pustule counts.

Such alterations in sebum secretion may be in part mediated through vasomotor changes in the skin. There are experimental data indicating that warming the skin increases sebum secretion under certain circumstances,[2, 4, 10, 11] and in states of depression, such as the "remorse" which was accompanied by decreased sebum output, there is a decrease in skin temperature.[6] Butcher and Coonin have shown that sebum becomes "highly viscous" at a temperature of 29 to 30° C.[3] It is possible that changes in sebum viscosity, resulting from changes in skin temperature, may have a significant effect on sebum flow, and hence on stasis and plug formation.

Of interest in this regard is the observation that acne vulgaris ordinarily is limited in its topographical distribution; occurring predominantly on the face, upper chest, and back. These are the "blush" areas of the body. There is evidence that the most active sebaceous glands are toward the head end of the human being,[5] which also may be a reason for its generally limited distribution.

In some additional exploratory experiments, however, sebum secretion and vascular alterations in the facial skin were found not to be simply or directly correlated.[11] Thus the nature of the relationship, if any, remains to be demonstrated.

It should be noted that, apart from the possibility discussed above (that the sebaceous glands are influenced by cutaneous vascular changes) the present data do not permit inferences regarding the mechanism of control of sebaceous gland activity. It is not possible to state to what extent, if any, there is *direct* nervous control of sebaceous gland function.

SUMMARY AND CONCLUSIONS

Thirty patients with acne vulgaris were studied to investigate the relation between stressful life situations, sebum secretion, and changes in the course of the disease.

In these patients, a characteristic emotional pattern was identified which consisted of episodes of "anger" followed by periods of "remorse" (guilt and depression). This was commonly set off by conflict with some figure of authority, usually a parent. The typical attitude statement was, "I wish they would let me alone and quit picking on me."

Day-to-day fluctuation in facial sebum secretion was no greater in acne patients than in subjects without acne.

A close correlation was found between life situations, giving rise to a characteristic affective pattern, and increase in the acne pustules of these patients.

Stressful interviews with acne patients were associated with increased sebum secretion when "anger" was elicited, and with decreased sebum secretion when the patients responded with "remorse."

There were no significant changes in sebum secretion during nonstressful interviews with acne patients, nor during stressful interviews in a control group of patients without acne.

It is suggested that abrupt, phasic alterations in the activity of the sebaceous glands may play an important part in comedo formation and hence in the pathogenesis of acne vulgaris.

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AN EVALUATION OF THE MEASUREMENT OF THE CARDIAC OUTPUT AND OF THE SO-CALLED PULMONARY BLOOD VOLUME BY THE DYE-DILUTION METHOD

JOSEPH T. DOYLE, M.D.,* JOSEPH S. WILSON, M.D., CHARLES LÉPINE, M.D., AND JAMES V. WARREN, M.D.

ATLANTA, GA.

DURING recent years there has been considerable reinvestigation of the measurement of the cardiac output by the dye dilution method and in the calculation of the so-called pulmonary blood volume. In many situations both in clinical and laboratory investigation, these measurements are of considerable interest. On the other hand, certain practical and theoretical difficulties have appeared. During the past three years, we have gained a considerable experience with this method and feel that a critical evaluation of this experience may be of some interest.

BACKGROUND

The method is based on the principle that if a marking substance be injected into a flowing liquid, the degree of dilution after adequate mixing is a measure of the volume of flow. In a closed system, the measurement must be made during the first circulation of the injected substance. The principle was first employed by Stewart in 1897.[1-3] Thirty years later, Hamilton and his co-workers revived and extended the method by using non-diffusing protein-binding dyes. The validity of the principle was established in glass models and in the heart-lung preparation. In the anesthetized dog a close correlation was found between the cardiac output measured by the dye-dilution and by the Fick methods. When applied to human beings, the method yielded values for the cardiac output regarded by contemporary critics as too high in comparison with those obtained by the then popular Grollman acetylene technique. As a consequence, the dye method fell into disrepute. Ten years later, the direct Fick method of estimating blood flow became applicable in human beings by venous catheterization of the heart. The values for the cardiac output derived by the Fick method were considerably higher than those found by the acetylene method. In 1948, Hamilton and Cournand published a study showing satisfactory agreement between the direct Fick and the dye methods.[5] The dilution method has also been extensively employed by Scandinavian investigators, in England by Kopelman, and by various groups in this country.[6-16]

The product of the blood flow and the mean circulation time gives the volume of blood between the point of injection of the marking substances and the point of collection. This measurement, termed "Q" by Stewart, comprehends the vascular territory between the point of injection in an arm vein and the point of arterial collection, and thus may be regarded as a crude index of the intrathoracic blood volume. In 1949, Ebert proposed a refinement wherein the dye is injected directly into a pulmonary artery through a cardiac catheter

From the Department of Medicine, Emory University School of Medicine and the Medical Service, Grady Memorial Hospital, Atlanta, Georgia.

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*Present address: Department of Medicine, Duke University School of Medicine, Durham, N. C.

rather than into a peripheral vein as in Hamilton's method.[17] It is thereby possible to measure the mean circulation time of the dye between the pulmonary artery and the point of arterial collection. The blood flowing during this period can be measured by either the direct Fick, or, in our opinion, preferably by the dye-dilution method.

Calculation of "Pulmonary" Blood Volume.—The volume of blood between the catheter tip and the arterial needle, then, is a crude measurement of the pulmonary blood volume, and includes presumably all moving blood in the lung, the left heart, the aorta, and all arteries temporally equidistant from the point of injection of the dyed blood (Fig. 1). This measurement has been extensively employed in this laboratory as part of a broad study of pulmonary hemodynamics.

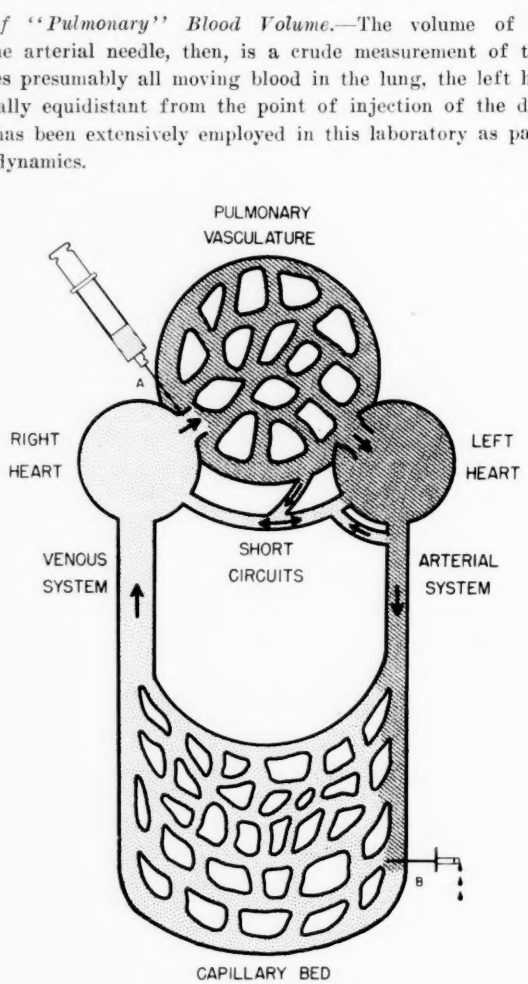

Fig. 1.—Schematic representation of the measurement of the "pulmonary" blood volume. The cardiac output and the mean circulation time from *A* to *B* are measured. The product of these values equals the volume of moving blood between the point of injection and all points temporally equidistant to point *B*.

The marking substances employed by Hamilton and others have been T-1824 and Brilliant Vital Red. These dyes are promptly fixed to the plasma protein and disappear only slowly from the vascular compartment. Although the use of these dyes in the estimation of the general blood volume has been severely criticized because of their escape into the lymph,[18] there is no reason to suppose that there is a significant loss during the few seconds

required to measure the blood flow. Recently, elegant methods for the continuous automatic recording of the dye-dilution curve have been devised.[19-21] Radioactive marking substances have been successfully used.[22]

The advantages and the limitations of the dye-dilution method will be discussed later.

METHODS

The subjects were hospital patients of either sex, ranging in age from the late teens to the mid eighties. They were fasting or had breakfasted lightly. Mild barbiturate sedation was occasionally employed. Observations were made with the patient in the recumbent position. A single or a double lumen catheter, the volume of which was accurately known, was introduced into the pulmonary artery. A Cournand needle was inserted into a brachial artery, occasionally a femoral.

A calibrated syringe containing a carefully measured amount of 5 per cent T-1824 (about 3.0 ml.) was attached to the catheter. The catheter was filled with the dye and then a rapid injection of the remainder of the dye was made. The injection time varied between 0.8 and 1.5 seconds, depending on the caliber and patency of the catheter and the pressure in the pulmonary artery. At the end of the injection, blood led through a short adapter from the Cournand needle was collected for two-second periods in small test tubes. The adapter and the test tube rack were operated by hand. In normal subjects the collecting period was twenty-two seconds. In subjects with a low cardiac output and a prolonged circulation time the collecting period was doubled. When the samples had been procured, the dye remaining in the catheter was flushed into the pulmonary artery with physiological saline solution. The oxygen consumption was then measured for a three-minute period and arterial and venous blood samples were in most cases simultaneously obtained for the direct Fick determination. Ten minutes after the dye injection a blood sample was obtained for estimation of the general blood volume. In some instances serial blood samples over a twenty-five-minute period were obtained for this purpose.

The tubes containing the two-second blood samples were centrifuged and from each 0.2 ml. serum was pipetted into clean tubes containing 1.8 ml. of physiological saline solution. The optical density of these solutions was read in 1.0 ml. cuvettes against a serum blank on a

TABLE I. CALCULATION OF MEAN CIRCULATION TIME AND CARDIAC OUTPUT FROM FIG. 2

T*	c†
4	0.0011
5	0.0033
6	0.0094
7	0.0270
8	0.0760
9	0.1060
10	0.1100
11	0.0980
12	0.0670
13	0.0420
14	0.0270
15	0.0170
16	0.0110
17	0.0068
18	0.0044
19	0.0026
20	0.0017
21	0.0011
Σ c =	0.6114
Σ c × t =	6.4666

(1) $C = \frac{0.6114 \times 10 \times 10}{0.285} = 215$ mg./L.

(2) $F_p = \frac{(3.0122 - 0.7632) \times 5 \times 60}{215} = 3.1$ L./min.

(3) $F_b = \frac{3.1}{100 - 36.4} = 4.9$ L./min.

(4) $MCT = \frac{6.4666}{0.6114} = 10.6$ sec.

(5) $PBV = \frac{10.6 \times 4.9 \times 1000}{60} = 866$ ml.

*t = time in seconds.
†c = optical density.

Beckman or a Coleman Junior spectrophotometer at a wave length of 620 mμ. The degree of hemolysis was estimated by reading each sample at 540 mμ.

The optical density of each sample was then plotted on a logarithmic ordinate against a linear time abscissa. Zero time was taken as the midpoint of the injection time and the mean collection time for each sample as the midpoint of the sampling interval, that is, one second (Fig. 2).

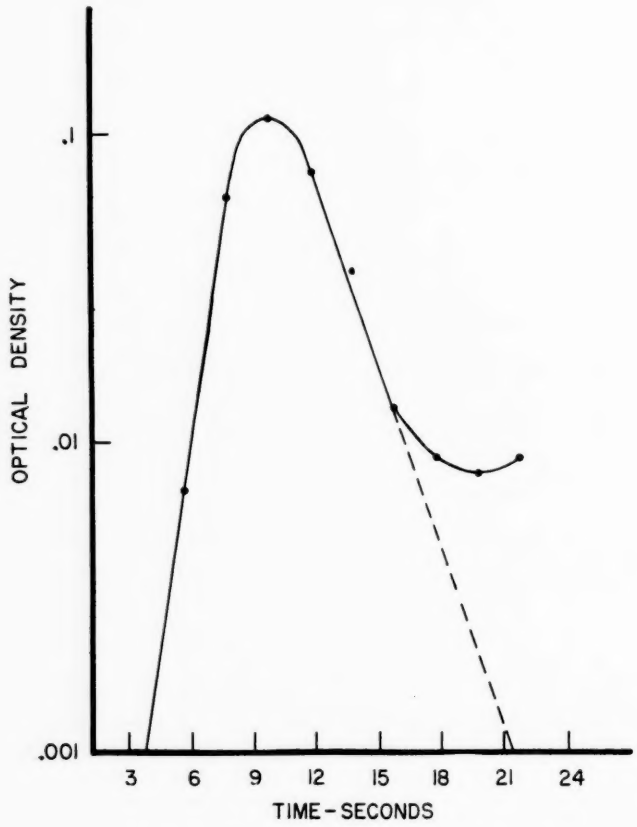

Fig. 2.—Dye-dilution curve. See Table II.

Calculation of Mean Circulation Time and Cardiac Output (Table I).—Thus, if the injection time were one second and the dye first appeared in the third tube, the first point was plotted at 5.5 seconds. The down stroke of the dye curve, as determined from the first several descending points before recirculation, was extrapolated as a straight line to the base line. From the time-concentration curve the optical density every second was read and the values cumulated. From this sum was derived the volume of plasma in which the injected dye had been diluted:

$$(1)\ C = \frac{\Sigma\, c \times 10 \times DF}{ODs}$$

where **C** = concentration of dye in mg. per liter
c = sum of dye concentration every second
DF = dilution factor (usually 1:10)
ODs = dye standardization factor: optical density of 1.0 ml. of T-1824 diluted 1:500

The plasma flow was then calculated:

$$(2)\quad Fp = \frac{(Sv - Cv) \times 5 \times 60}{C}$$

where Fp = plasma flow in liters per minute
Sv = syringe volume in milliliters
Cv = catheter volume in milliliters
5 = milligrams dye in 1.0 ml. of T-1824
60 = seconds in 1 min.

The blood flow, that is, the cardiac output, was obtained from the hematocrit reading:

$$(3)\quad Fb = \frac{Fp}{100 - Het}$$

where Fb = blood flow in liters per minute
Het = hematocrit reading

The average time required for a particle of dye to pass from the tip of the catheter to the arterial needle was calculated as follows:

$$(4)\quad MCT = \frac{\Sigma\,(c \times t)}{\Sigma\,c}$$

where MCT = mean circulation time
c = concentration of dye at 1 sec. intervals
t = cumulative 1 sec. intervals

The volume of blood between the catheter and the arterial needle was then derived:

$$(5)\quad PBV = \frac{MCT \times Fb}{60}$$

where PBV = pulmonary blood volume in milliliters

The general blood volume was finally calculated:

$$(6)\quad PV = \frac{Sv \times ODs \times 500}{OD_{10}}$$

where PV = plasma volume in milliliters
500 = volume in milliliters to which 1.0 ml. of T-1824 was diluted to obtain ODs
OD_{10} = optical density of unknown serum

$$(7)\quad GBV = \frac{PV}{100 - Het}$$

where GBV = general blood volume in milliliters

The cardiac output and the pulmonary and the general blood volume were reduced to values per square meter by dividing these values by the body surface in square meters. The ratio of the pulmonary to the general blood volume was expressed as the percentage PBV/GBV.

In a small group of subjects in whom only 0.3 ml. of T-1824 was injected, the blood samples were collected every second and the optical density of the undiluted serum was measured in microcell cuvettes. Except for the smaller dose of dye, there appeared to be no advantage over the previously outlined method.

TABLE II

	MEAN	STANDARD DEVIATION
Mean circulation time (sec.)	11.5	± 2.7
Cardiac index (dye) (L./min./M.²)	3.2	± 0.2
Cardiac index (Fick) (L./min./M.²)	3.5	± 0.3
Pulmonary blood volume (ml./M.²)	634.0	±122.0
General blood volume (ml./M.²)	2775.0	±147.0
PBV/GBV %	23.0	± 4.0

TABLE III

	I			II		
	MEAN	STANDARD DEVIATION	STANDARD ERROR	MEAN	STANDARD DEVIATION	STANDARD ERROR
Mean circulation time (sec.)	12.2	± 2.2	± 0.68	12.6	± 2.3	± 0.89
Cardiac index (Dye) (L./min./M.²)	3.2	± 0.7	± 0.23	3.2	± 1.0	± 0.32
Cardiac index (Fick) (L./min./M.²)	3.8	± 0.8	± 0.24	3.5	± 0.4	± 0.23
Pulmonary blood volume (ml./M.²)	630.0	±134.0	± 42.0	642.0	±195.0	± 65.0
General blood volume (ml./M.²)	2896.0	±123.0	±116.0	2766.0	±370.0	±123.0
PBV/GBV %	22.0	± 4.0	± 1.0	23.0	±371.0	± 2.0

RESULTS

The correlation between the cardiac output measured by the dye and the Fick methods is shown in Fig. 3. This figure includes 152 normal as well as abnormal subjects studied over the first eighteen months of this project. The only data excluded are those instances in which it was impossible to construct a dye-dilution curve. This difficulty occurs rarely in normal subjects, and in perhaps 10 per cent of the subjects with severe congestive heart failure. In these latter individuals and in the case of shunts in or near the heart, recircu-

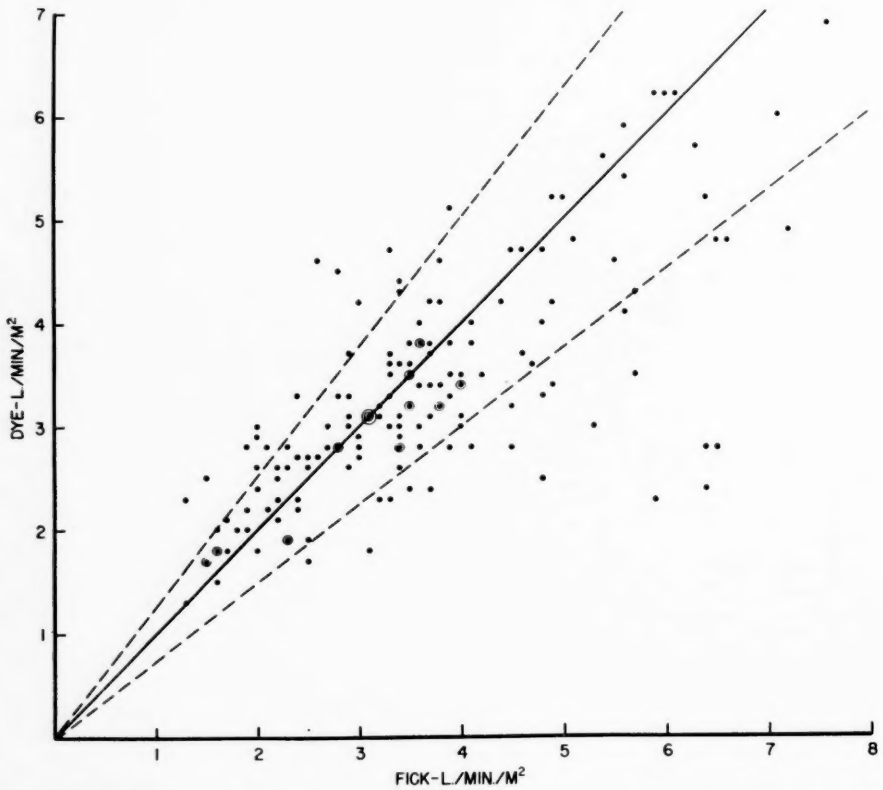

Fig. 3.—Correlation of 152 approximately simultaneous measurements of the cardiac output by the dye-dilution and the direct Fick methods. The dotted lines represent a ± 25 per cent variation. r = 0.73.

lation renders it impossible to extrapolate accurately the descending limb of the dye curve. Undoubtedly, some of the charted cases represent random technical errors, the identification of which is impossible. Nonetheless, the majority of determinations show an agreement with ± 25 per cent. The correlation coefficient is 0.73.

Comparison of Nearly Simultaneous Measurements of the Cardiac Output by the Dye-Dilution and the Fick Methods in Both Normal and Abnormal Subjects.—The findings in 53 normal, recumbent, resting individuals are shown in Table II.

In 10 of these individuals the same measurements were repeated as soon as possible. Presumably the subjects were in the same hemodynamic status during both sets of measurements. These data are presented in Table III. It is felt that the standard errors and deviations are within acceptable limits.

DISCUSSION

These data are in general agreement with those of Hamilton,[5] Johnson,[7] and Kopelman.[11] The errors inherent in the method include the possibilities of incomplete mixing of the dye; the escape of the dye from the vascular compartment; regional variations in the ratio of the red cells to the plasma; the sequestration of blood from the general circulation; and purely technical errors and difficulties such as turbidity of the serum, hemolysis, inaccuracies in the blood collection, pipetting, dilution, and so forth. Unquestionably the source of the greatest error is the difficulty in the recognition of the early recirculation of the dye. Such recirculation is identified by the departure of the dilution curve from the straight line of the descending limb (Fig. 2). It is apparent that a considerable increase in the recirculating dye may occur before this break in the line becomes visible. Sutton and associates[23] have shown that when a radioactive marker is injected into the pulmonary artery, radioactivity is demonstrable in the right ventricle as early as eight seconds later. It should also be pointed out that a certain element of subjectivity enters into the construction of the dye-dilution curve. Our experience has shown that some degree of skill in drawing the curve must be acquired before consistently satisfactory checks with the Fick method can be obtained. It should also be noted that the dye-dilution and the Fick procedures cannot be done simultaneously. Moreover, the dye procedure measures blood flow over only 20 to 30 heart cycles, while the Fick procedure measures an average value for flow over a two- or three-minute period. Despite these hazards the agreement between the dye and the Fick methods is reasonably good.

The dye-dilution method possesses certain distinctive advantages. The cooperation of the subject is not essential, whereas it is in the direct Fick method. Therefore the blood flow can be measured easily and rapidly in various stress situations. Since the oxygen consumption does not enter into the calculation, it is possible to measure blood flow during unsteady states such as acute hypoxia.[13] Massive early recirculation of the dye occurring through septal defects or a patent ductus arteriosus produces a saddleback dilution curve of diagnostic value. Instrumental methods of recording the time-concentration curve will make it possible to measure a large number of cardiac outputs in rapid succession.[24]

The principle employed for the calculation of the volume of blood between the catheter tip and the arterial collecting needle from the product of cardiac output per second and the mean circulation time is, for simple systems, mathematically irreproachable. The accuracy of such a measurement in complex biological systems is obviously limited by the accuracy with which the flow and the mean circulation time can be measured. Table III indicates that a

methodical error of ± 10 per cent may be anticipated, or a volume of about ± 100 milliliters. A priori, it seems likely that volume shifts in the lungs considerably smaller than 100 ml. may be of physiological importance. We would therefore agree with Johnson[7] that a change in the pulmonary blood volume of 20 to 30 per cent is required to achieve significance. The pulmonary blood volume cannot be correlated with the pulmonary arterial or the pulmonary capillary mean pressures; with the right ventricular work, with the pulmonary vascular resistance, or with the vital capacity. Our data, like Johnson's, show a good correlation between the pulmonary blood volume and the stroke index. Such a correlation is inherent in the calculation of the pulmonary blood volume. Accordingly, Johnson's conclusion that the cardiac output is determined by the dependent variable of the pulmonary blood volume cannot be accepted. Furthermore, as is shown in Table VI and in unpublished studies on the effects of l-epinephrine on the circulation, it is quite possible to alter the cardiac output without significantly altering the pulmonary blood volume.

The constancy of the ratio of the pulmonary to the general blood volume under a considerable variety of circumstances is rather remarkable, and would appear to be evidence against the concept of the lung as a blood reservoir. Thus, if the general blood volume suddenly be expanded by the rapid infusion of a liter of physiological saline solution,[12] there is no redistribution of the circulating blood volume (Table IV).

TABLE IV. RAPID INTRAVENOUS INFUSION OF PHYSIOLOGIC SALINE SOLUTION IN TWELVE SUBJECTS

	CONTROL	INFUSION
Mean circulation time (sec.)	12.4	11.0
Cardiac index (dye) (L./min./M.^{2})	2.9	3.6
Cardiac index (Fick) (L./min./M.^{2})	3.6	4.3
Pulmonary blood volume (ml./M.^{2})	560.0	630.0
General blood volume (ml./M.^{2})	2700.0	3080.0
PBV/GBV %	21.0	20.0

Likewise, in chronic congestive heart failure the expanded blood volume appears to be symmetrically distributed in the lesser and in the greater circulations (Table V).

TABLE V. FOURTEEN SUBJECTS WITH CONGESTIVE HEART FAILURE

Mean circulation time (sec.)	24.9 ± 5.2
Cardiac index (dye) (L./min./M.^{2})	2.1 ± 0.7
Cardiac index (Fick) (L./min./M.^{2})	2.2 ± 0.8
Pulmonary blood volume (ml./M.^{2})	892 ± 257
General blood volume (ml./M.^{2})	3575 ± 206
PBV/GBV %	25 ± 5

Moreover, the same failure to alter the ratio of the pulmonary to the general blood volume has been found in a small group of subjects in whom the effective circulating blood volume was reduced by the application of venous occluding tourniquets to the thighs or by phlebotomy.[25] Finally, despite moderate increases in the cardiac output and very large increases in the pulmonary

vascular resistance induced by hypoxia, no change in the pulmonary blood volume can be detected (Table VI). The pulmonary blood volume and the ratio of the pulmonary to the general blood volume are normal in chronic lung disease.

TABLE VI. HEMODYNAMIC EFFECTS OF ACUTE HYPOXIA IN EIGHT NORMAL SUBJECTS

	CONTROL	HYPOXIA
Mean circulation time (sec.)	11.9	9.7
Cardiac index (dye) (L./min./M.²)	3.4	4.4
Cardiac index (Fick) (L./min./M.²)	3.5	
Pulmonary blood volume (ml./M.²)	660.0	670.0
General blood volume (ml./M.²)	2810.0	
PBV/GBV %	24.0	24.0

Experimentally, the ratio of the pulmonary to the general blood volume can be strikingly reduced during the Flack maneuver.[25] Clinically it is only when the sharply contrasting hemodynamic abnormalities of tight mitral stenosis and free tricuspid insufficiency are compared that an equally significant difference in the ratio of the pulmonary to the general blood volume becomes apparent (Table VII).

TABLE VII. COMPARISON OF SIX CASES OF MITRAL STENOSIS WITH FIVE CASES OF TRICUSPID INSUFFICIENCY

	MITRAL STENOSIS	TRICUSP. INSUFF.
Mean circulation time (sec.)	24.6	16.3
Cardiac index (dye) (L./min./M.²)	2.1	2.5
Cardiac index (Fick) (L./min./M.²)	1.8	2.3
Pulmonary blood volume (ml./M.²)	860.0	670.0
General blood volume (ml./M.²)	3120.0	3530.0
PBV/GBV %	28.0	19.0

The small mitral orifice distal to the catheter presumably accounts for the greatly prolonged mean circulation time and the large volume of blood impounded in the lungs and the left atrium. In two instances, the pulmonary blood volume was found to rise tremendously with exercise, and severe dyspnea developed. Several of these subjects were estimated at valvotomy to have mitral orifices of less than 1 sq. cm., confirming the area calculation found by the Gorlin formula. The presence of severe, long-standing mitral stenosis with associated salt and water retention appears to be the best explanation of these findings, which are in contrast with those of Ebert[17] and Tompkins,[26] but are in agreement with Kopelman's data derived from cardiac catheterization.[11] In tricuspid insufficiency it is presumed that since the catheter lay beyond the point of copious reflux of blood into the venous system, the mean circulation time was fairly normal. The finding of a relatively normal pulmonary blood volume in these cases agrees with the clinical experience that the evolution of tricuspid insufficiency relieves the symptoms of pulmonary congestion.

SUMMARY AND CONCLUSIONS

The dye-dilution method of Stewart and Hamilton is a useful technique of measuring the cardiac output. In 152 consecutive measurements of the cardiac

output by the dye-dilution and the direct Fick methods, the correlation coefficient was 0.73, while in a smaller group the reproducibility of both methods was shown to be good.

As a corollary of this conclusion, the estimation of the so-called pulmonary blood volume is likewise a reproducible measurement. It appears, however, that an anticipated error of ± 10 per cent renders the measurement insensitive to small but presumably significant changes in the pulmonary blood volume.

Evidence is presented which suggests that blood is not sequestered in the lungs. Thus, the acute expansion or reduction of the effective blood volume does not alter the ratio of the general to the pulmonary blood volume. Similarly, the pulmonary hypertension induced by hypoxia does not alter this ratio.

The advice and criticism of Dr. John L. Patterson, Jr., is gratefully acknowledged.

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EFFECT OF PROLONGED ADMINISTRATION OF RADIOACTIVE ZINC^{65} ON THE PANCREAS

JAMES W. SHERRILL, M.D., AND ARNE N. WICK, PH.D.
LA JOLLA, CALIF.

CONSIDERABLE indirect evidence has led to the belief that zinc is an integral part of the insulin molecule. Scott[1] and Scott and Fisher[2] crystallized insulin as the zinc salt and reported that under their conditions of crystallization insulin and zinc combined in a constant ratio. Purification studies by other investigators[3, 4] have also indicated the natural occurrence of zinc in insulin.

In a study on the turnover of zinc, Sheline and associates[5] injected tracer amounts of the labeled isotope into mice and dogs. Of the various tissues examined, the pancreas was found to have the highest concentration of the radioactive zinc. Drinker and associates,[6] in a study on the long-continued ingestion of nonradioactive zinc by cats and dogs, reported that the only damage detected by the daily administration of 175 to 1,000 mg. of zinc oxide for periods varying from three to fifty-three weeks were fibrotic changes in the pancreas of the cat. Because of the close association of zinc, insulin, and the pancreas, the administration of radioactive zinc,[65] a strong beta emitter, over an extended period could result in the destruction of the insulin-producing cells of the pancreas. We have tested this hypothesis by administering zinc[65] to rats over a six-month period. The criteria used for the diabetic symptoms were urine and blood sugar determinations and body weight.

TABLE I. TERMINAL BLOOD SUGAR VALUES

GROUP	TEST SUBSTANCE	ANIMAL NUMBER	BLOOD SUGAR (MG/%)
1	Zn^{65} acetate	1	161
		2	150
		3	176
		4	163
		5	162
2	Inactive Zn acetate	6	156
		7	146
		8	148
		9	170
		10	156
3	Zn^{65} acetate	11	127
		12	141
		13	159
		14	161
		15	150
4	Inactive Zn acetate	16	156
		17	147
		18	160
		19	155
		20	152

From the Scripps Metabolic Clinic, La Jolla, Calif.
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MATERIALS AND METHODS

Male albino rats weighing 160 to 180 Gm. were used. The animals were divided into four groups of 5 each. Two groups of the animals were injected intraperitoneally with 10 microcuries of radioactive zinc^{65} acetate* each week except Group 1 which received 36 microcuries for the first forty-one days and then shifted to the lower dosage level. The remaining two groups were used as controls and injected with the corresponding weight of the inactive zinc acetate. The animals were maintained on a stock diet of Purina Laboratory Chow except for the diabetic test periods. Urine and blood glucose determinations for the diabetic symptoms were carried out on each animal at approximately two-month intervals. In order to accentuate the diabetic symptoms, the animals were placed on a high carbohydrate diet for 14 days before collecting 2 successive 24 hour urine specimens and taking a blood sample for glucose determinations. Groups 1 and 2 were maintained for 78 days; groups 3 and 4 for 168 days. Weekly weighings of the animals were recorded.

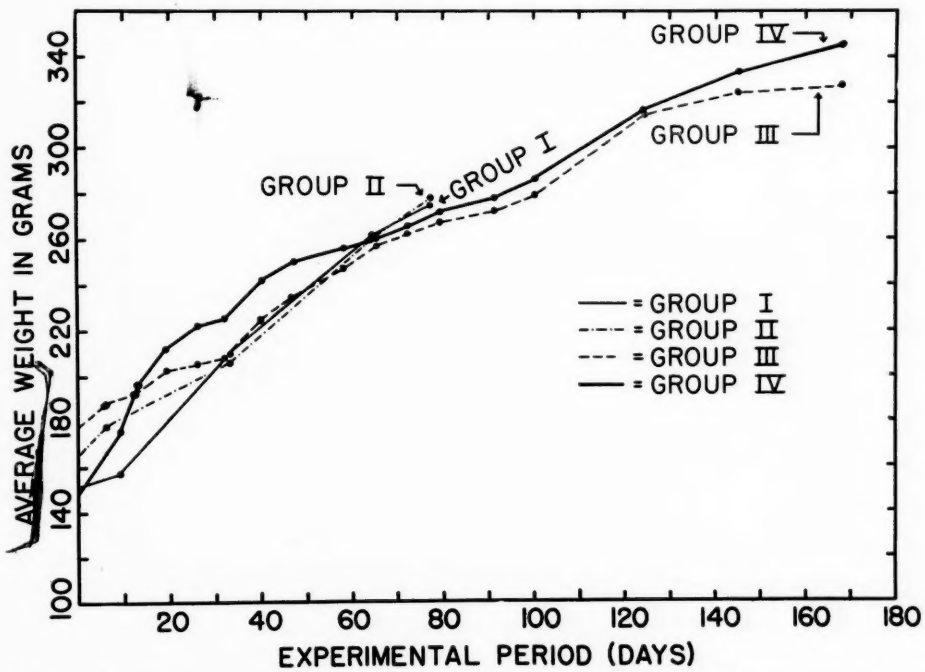

Fig. 1.—Growth curve of rats. Groups 1 and 3 injected weekly with Zn^{65} acetate; Groups 2 and 4 injected weekly with inactive Zn acetate.

RESULTS

Fig. 1 shows the average weights for the animals during the experimental period. The weight gain for the rats injected with the isotope and the inactive zinc was approximately the same. The terminal blood sugar concentration for the individual rats are given in Table I. Although the blood sugar values are higher than normal due to the high carbohydrate content of the test diet, there is no appreciable difference in the values for the animals receiving radioactive or inactive zinc. All of the animals excreted less than 2 mg. of reducing substances calculated as glucose over a twenty-four hour period.

*The zinc^{65} acetate was purchased from Tracerlab, Inc., Boston. One millicurie was equivalent to 70 mg. of the salt.

DISCUSSION

The weekly administration of 10 microcuries of a tracer dose of radioactive zinc to rats failed to produce in these experiments the diabetic symptoms of loss of body weight, glycosuria, or a high blood sugar. There is no doubt that the pancreas of each animal was exposed to the zinc radiation. Montgomery and associates[7] have presented evidence that the pancreas is concerned with the metabolism of zinc. These authors report that after a single injection, approximately 11 per cent of the injected zinc^{65} was eliminated by way of the external secretion of the pancreas in fourteen days. The quantity of zinc^{65} used in these experiments, which corresponds to 2 to 3 millicuries for a 60 kilogram man, per injection, was sufficient to make the rats highly and constantly radioactive when tested by the external application of a Geiger counter. Perhaps more frequent injections, or larger doses, would have had a measurable physiological action.

SUMMARY

Radioactive zinc^{65} acetate was administered weekly to rats for a six-month period. No diabetic symptoms, such as loss of body weight, glycosuria, or high blood sugar resulted from the zinc radioactivity.

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EXCRETION OF WATER AND ELECTROLYTES IN PATIENTS WITH ESSENTIAL HYPERTENSION

WILLIAM A. BRODSKY, M.D., LOUISVILLE, KY., AND CINCINNATI, OHIO, AND
HARRY N. GRAUBARTH, M.D., CINCINNATI, OHIO

INTRODUCTION

ALTERATIONS of renal function in hypertensive diseases have been recognized by many investigators from different points of view. While early workers observed defects in the renal capacity for water concentration and salt excretion, later investigators have demonstrated varying degrees of renal ischemia. In these and in other reports only fragmentary information concerning the relation between water and solute excretion and renal hemodynamics is available.

Koranyi[1] and others,[2] by means of cryoscopic measurements, found that hypertensive patients were unable to concentrate their urine to normal levels. Data presented[1] showed that the ratio of total osmolarity to chloride in the urine was higher than that of normal subjects, a finding regarded as compatible with increased retention of salt. Widal and Javal[43] and Ambard and Beauvard[44] also found evidence of salt retention in different varieties of Bright's disease. More recently, however, Farnsworth and Barker[4] observed under conditions of water diuresis that the U/P ratios of chloride in hypertensive disease were higher than normal.

After the introduction of various clearance techniques, attention shifted from the excretory to the hemodynamic aspects of renal function. While some[7, 13] detected decreases in renal blood flow with high filtration fraction and diminished capacity for the tubular excretion of diodrast, others[14] failed to find a reduction of blood flow in the hypertensive kidney. Goldring and associates[15] and Smith[16] interpret as evidence of renal ischemia the finding of a low ratio of diodrast clearance to diodrast Tm.

Interpretation of the aforementioned studies is obscured by the fact that essential hypertension may be complicated by varying degrees of cardiac and renal insufficiency.[17, 18] Such complications may be expected to affect the rates of glomerular filtration, renal plasma flow, and excretion of water and solutes.

The purpose of the present report was to assess osmotic as well as hemodynamic activities of the hypertensive kidney, and, if possible, to dissociate the effects of hypertension per se, from those of cardiac or renal insufficiency.

From the Department of Pediatrics and Institute for Medical Research, University of Louisville, Louisville, Ky., and the Children's Hospital Research Foundation, University of Cincinnati, Cincinnati, Ohio.

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It appeared that the technique of osmotic diuresis might provide a useful means of accomplishing such an evaluation.

Osmotic diuresis with mannitol loading was produced in six subjects with essential hypertension. The rates of urine flow and solute excretion before and during loading as well as those of glomerular filtration and renal plasma flow were studied.

MATERIALS AND METHODS

Patients of the study were 4 women and 2 men with essential hypertension. As far as was known, they were and had been free of edema or other complicating symptoms such as dyspnea, orthopnea, basal râles, enlarged liver, elevated venous pressure, headache, visual disturbances, or nocturia. Urinary cultures yielded no organisms and I.V. pyelograms were within normal limits. No clinical history referable to urinary tract obstruction or infection was obtained. Patients had been observed by the Hypertensive Clinics at both the Cincinnati and Louisville General Hospitals. The women were 17, 48, 48, and 41 years of age, and the men 39 and 58 years of age. Table I provides essential clinical data obtained just prior to the testing. On the morning of the test, after 1 or 2 preliminary periods of urine collection, 25 per cent mannitol was administered intravenously as a priming solution in amounts calculated to add 20 milliosmoles per liter of distribution volume. The volume of distribution of mannitol was assumed to be equivalent to 20 per cent of the body weight. During infusion of maintenance solutions (in amounts of 1 per cent of the priming dose per minute), urine and blood were collected and analyzed for osmotic activity, mannitol, urea, Na, K, and Cl by techniques described elsewhere.[19] In all, six tests comprising 41 collection periods were performed. Thirty-one of the periods were collected during mannitol loading.

The patients experienced no untoward symptoms other than mild thirst throughout all tests. In no instance did the level of blood pressure change during the loading procedure.

TABLE I. PERTINENT CLINICAL INFORMATION ON THE SIX HYPERTENSIVE PATIENTS OF THE PRESENT STUDY

NAME	AGE	SEX	COLOR	B.P.	SP. GR.	PSP. (%)	B.U.N. (MG./100 c.c.)	YEAR HYPERTENSION FOUND	TEAC RESPONSE
J.W.	58	M	C	210/140	1023	75	17	—	—
L.B.	48	F	C	212/146	—	70	7	1945	—
S.S.	48	F	C	156/110	1022	60	14	1937	good
C.W.	39	M	C	172/116	1024	—	11	1944	good
E.K.	17	F	W	172/124	1030	70	11	1950	moderate
S.T.	41	F	C	210/130	1014	—	12	1945	

RESULTS

Urinary Flow and Osmotic Activity.—Table II presents for the six patients values for urinary flow and osmotic activity during preliminary periods of urine collection. Data represent averages of two to three periods on each patient. Included for comparative purposes are mean values with their standard deviations, for similar data from 16 normal "hydropenic" subjects.[20]

It can be seen that 4 of the 6 patients were excreting urine at higher than normal rates of flow, 0.9 to 1.70 ml. per minute per 1.73 squaremeter, with submaximal osmolarities of 557 to 713 milliosmoles per liter. One patient, S. T., attaining a minimal rate of flow, failed to reach a maximal level of osmotic activity, while another, E.K., responded normally. The results are in harmony

with those of others who have described obligatory polyuria and concentration defects in hypertensive disease.[1, 3, 17] With regard to the excretion of individual solutes, the concentration of urea was lower, and that of Na and Cl the same or higher (patient E.K.) than the mean values observed in normal subjects. Rates of Na and Cl loss reached levels of almost two and one-half times those of normal subjects, an observation indicating that the hypertensive kidney, under hydropenic conditions, has a reduced capacity for conservation of salt.

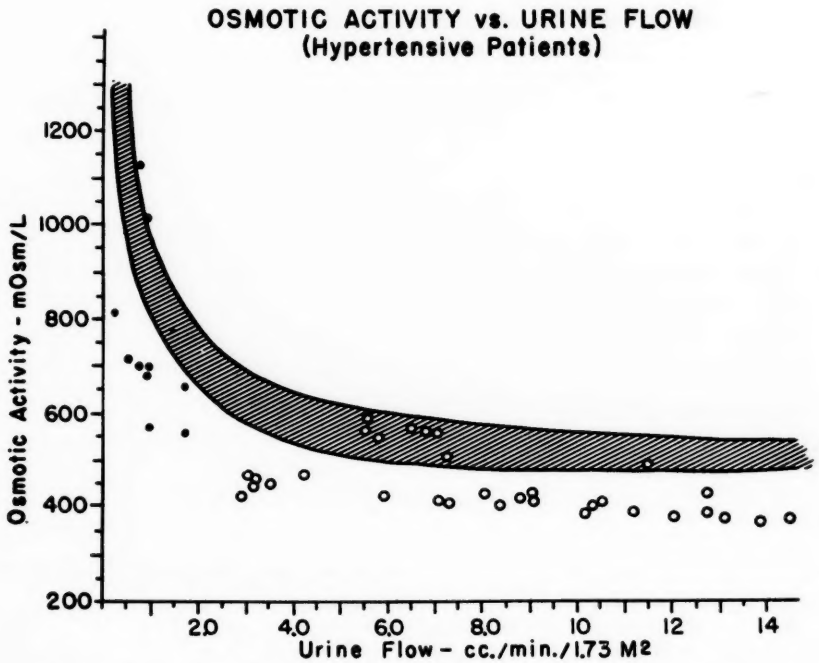

Fig. 1.—Effective osmotic activity versus urine flow in hypertensive patients. The shaded area represents the entire range of values obtained from normal subjects under the same conditions.[17]

Fig. 1 presents a plot of the values for osmotic activity versus flow, during preliminary and loading periods of urine collection. Data presented comprise a total of 41 periods, of which 31 represent those obtained during loading. It is apparent that, over a wide range of urine flows, osmotic activity fell to values of 375 to 469 milliosmoles per liter as compared to normal values of 510 to 570 during loading.[20] Two of the patients, E.K., and L.B., responded in a manner identical to that of normal subjects.

Fig. 2 depicts a plot of the values of urinary flow versus effective osmotic load (effective osmotic load equals rate of urine flow times effective osmotic activity). It can be seen that most of the data group themselves at a higher level than those of normal subjects. For a given solute load, patients with hypertension excreted urine at a rate of about one and one-half times that of normal subjects.

TABLE II. URINE FLOW AND EFFECTIVE OSMOTIC ACTIVITY IN THE URINE OF HYPERTENSIVE PATIENTS UNDER PRELOADING HYDROPENIC CONDITIONS

PATIENT	FLOW (C.C./MIN./-1.73M²)	OSMOTIC ACTIVITY (MOSM./L.)	UREA N (MOSM./L.)	Na (MEQ./L.)	K (MEQ./L.)	Cl (MEQ./L.)
J.W.	0.98	698	149	252	26.5	209
C.W.	0.91	713	255	104	120	166
	1.17	694	221	148	107	181
E.K.	0.86	1026	247	307	75	346
	0.80	1131	273	—	—	354
L.B.	1.02	567	233	110	25	102
	0.96	677	298	137	25	122
S.S.	1.70	557	207	129	21	118
	1.65	657	284	131	31	123
S.T.	0.33	806	255	197	70	293
NORMAL SUBJECTS MEAN VALUE	0.60	1104	405	161	—	215
STANDARD DEVIATION	0.24	284	78	60	—	68

Data from Figs. 1 and 2 signify, in 4 of 6 hypertensive patients, the presence of a concentration defect over a wide range of urine flows, 0.33 to 14 ml. per minute. On the basis of previous discussions,[19, 21] one may, by inference, localize the site of such defects to the most distal portion of the renal tubule.

In the process of formation of hypertonic urine, a water economy must be effected. Previous work[20, 22] has shown that renal water economy may be quantitated from the equation, $\frac{\text{effective osmotic load}}{\text{plasma osmotic activity}}$ − urine volume = water economy H_2O_{ec}. Fig. 3 is a plot of the values for water economy versus urinary flow in the hypertensive patients. The shaded area represents the range of values of normal subjects.[20] It can be seen that values of water economy from 4 of the patients were less than one-half those of normal subjects.

Solutes in Plasma and Urine.—Table III presents, for 25 periods of urine collection during loading, values for concentrations of solutes in urine and plasma. In plasma, the mean concentration of mannitol was raised to 22.6 milliosmoles per liter, while the calculated osmolarity increased by an average of 7 milliosmoles per liter over the preloading level. Changes induced in the plasma of hypertensive patients by mannitol loading were practically identical to those of normal subjects under the same conditions.[20]

In the urine, mannitol accounted for 30 per cent of the total osmolarity in 4 of the patients. For the entire group of patients, the concentration of mannitol reached a mean value of 196, as compared to the normal mean of 269 milliosmoles per liter.[20] Urinary sodium, higher than normal in 5 patients, reached a value as high as 139 meq. per liter (J.W., period 1). The mean concentration of sodium for the 6 patients was 93.2, while that of normal subjects was 68.6 meq. per liter. Values for urinary chloride were, in most cases, similar to or slightly less than those of sodium. Potassium, at concentrations of 5-12 meq. per liter, accounted for no more than 3 per cent of urinary solutes. Solutes determined

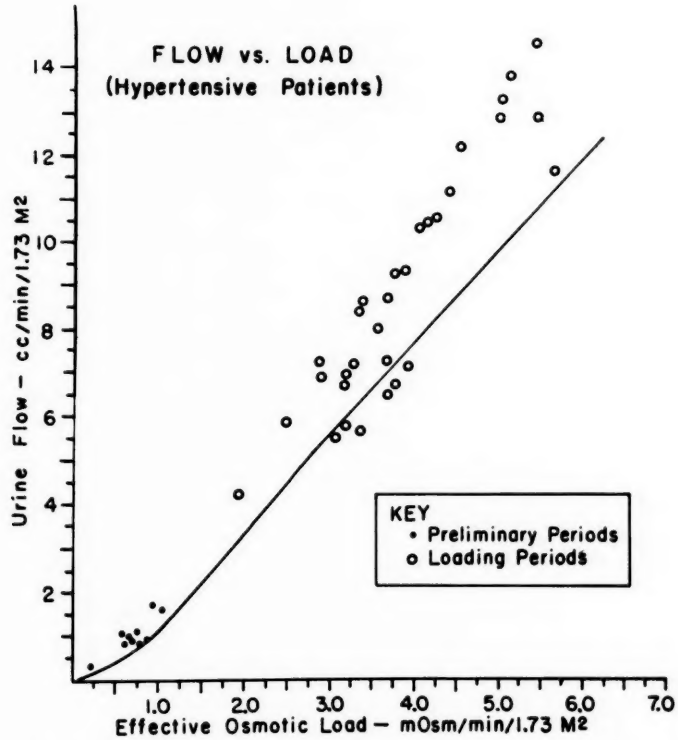

Fig. 2.—Urinary flow versus effective osmotic load. The solid line is taken from data obtained previously on normal subjects.[17]

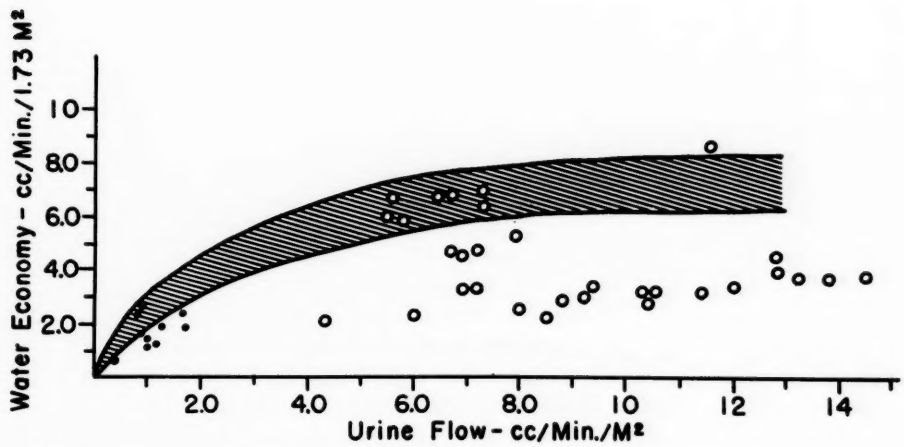

Fig. 3.—Water economy versus urine flow in hypertensive patients. Shaded area equals range of normal values.

TABLE III. URINE FLOW AND SOLUTE CONCENTRATIONS IN URINE AND PLASMA DURING MANNITOL DIURESIS OF HYPERTENSIVE PATIENTS

PATIENT	PERIOD	URINE FLOW (C.C./MIN./-1.73M²)	OSMOTIC ACTIVITY (MOSM./-L.)	MANNITOL (MOSM./-L.)	UREA (MOSM./-L.)	Na (MEQ./-L.)	Cl (MEQ./-L.)	K (MEQ./-L.)
L.B.	1 U	7.30	510	239	45.2	91.5	81.4	8.28
	P		277.4	18.7	5.48	140	102	3.69
	2 U	5.64	596	271	50.3	84.5	75.8	8.63
	P		273.8	17.4	4.94	139	104	3.72
	3 U	5.50	570	309	54.8	72.5	65.2	8.68
	P		272.6	17.6	4.86	138	104	4.20
	4 U	5.84	554	276	51.3	84.0	75.4	8.68
	P		—	—	—	—	—	—
S.S.	1 U	12.8	394	129	34.9	94.0	82.8	6.50
	P		300.7	28.4	9.3	146	102	3.43
	2 U	14.5	378	137	30.4	96.8	85.2	5.20
	P			27.2	9.7			3.75
	3 U	13.2	384	133	35.5	88.0	81.4	5.61
	P			27.2	9.3		107	
	4 U	13.8	375	134	30.3	88.4	82.8	5.80
	P			27..8	—			
	5 U	12.0	382	146	33.2	88.8	80.2	6.16
	P			28.2	—	142	102	4.03
J.W.	1 U	12.8	426	133	37.5	139	107	6.5
	P		312.6	20.4	5.36	158	108	4.14
	2 U	8.79	416	144	29.8	124	99.2	5.2
	P		314.1	21.7	5.72	158	110	4.20
	3 U	8.48	403	143	26.5	123	97.2	5.0
	P		315.8	23.3	5.66	158	109	4.50
	4 U	10.4	405	144	25.5	120	95.6	5.0
	P	—	—	—	—	—	—	—
C.W.	1 U	7.96	454	172	32.5	77.0	78.2	10.1
	P		275.3	21.1	4.04	138	104	4.46
	2 U	6.89	463	209	33.2	77.0	74.8	11.0
	P		275.3	21.1	4.04	138	101	4.48
	3 U	7.24	455	198	32.2	62.0	75.4	11.2
	P		273.7	21.3	4.21	137	101	4.46
	4 U	6.74	469	205	33.8	75.5	72.0	16.2
	P		273.7	21.3*	4.21*	137*	101	
E.K.	1 U	7.12	556	251	36.7	100	107	11.7
	P		280.8	22.1	4.58	140	106	4.90
	2 U	6.68	564	272	36.5	91.0	96.8	11.4
	P			24.0	—	140	—	4.67
	3 U	6.50	572	284	38.1	91.0	96.0	11.0
	P			21.5	—	140	107	4.70
S.T.	1 U	11.4	390	172	21.8	98.8	85.7	7.60
	P		306.6	21.4	4.25	155	104	3.81
	2 U	10.5	407	173	22.9	98.7	82.4	8.76
	P		309.6	23.2	3.61	156	102	3.88
	3 U	9.24	412	200	24.0	91.5	79.8	10.0
	P		308.7	22.0	4.01	156	106	3.75
	4 U	9.31	420	205	24.6	88.5	77.5	8.04
	P		305.4	21.0	3.88	155	105	3.25
	5 U	8.04	424	218	25.1	83.5	73.4	9.80
	P		313.0	22.0	3.82	158	102	4.11
Hypertensive* (25)	U	9.15±0.55	455±14	196±11	33.9±1.8	93.2±1.1	84.2±2.2	
Normal (41)	U	7.37±0.18	540± 5	269± 5	56.1±1.5	68.6±1.5	68.2±1.3	

Numbers in parentheses indicate the number of periods of urine collection in each group of patients.

*Mean values with standard errors for water and solute excretion in urine of hypertensive and normal subjects during osmotic diuresis.

comprised over 90 per cent of the total osmolarity. Phosphate, bicarbonate, and sulfate were not determined.

The findings of higher than normal amounts of sodium and chloride in the urine of hypertensive patients are similar to those reported elsewhere[1-6] under conditions of water diuresis. All patients had been on free diets without salt restriction for at least two weeks prior to the tests.

In Fig. 4 is presented a plot of values of sodium excretion per minute versus effective osmotic load, during 26 periods of mannitol loading in the hyper-

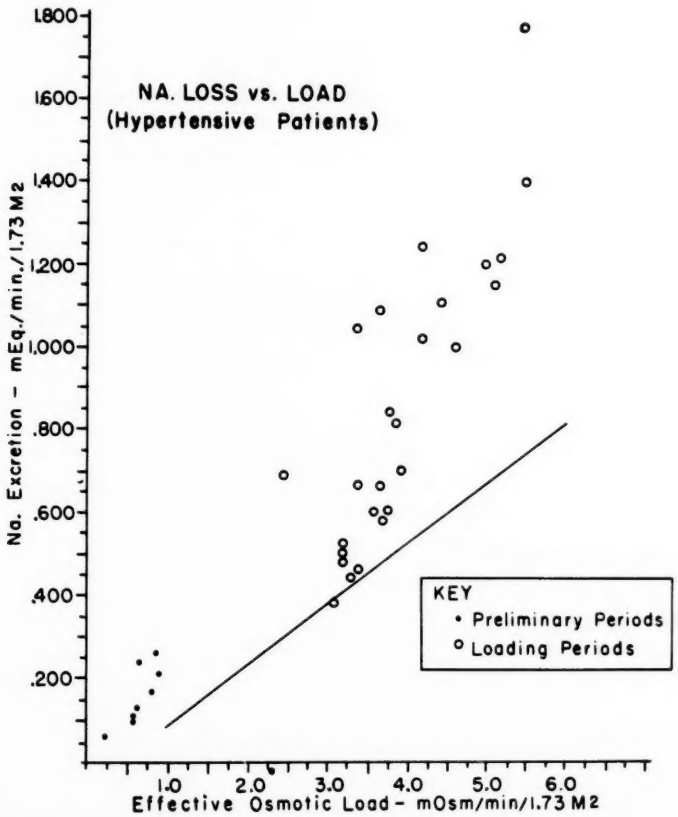

Fig. 4.—Sodium excretion versus effective osmotic load. Solid lines represent calculated linear regressions for values of normal subjects.

tensive subjects. The pattern of chloride loss versus load, not presented, was practically identical to that of sodium loss versus load. Minute losses of sodium and chloride were high, reaching values of as much as 1.8 and 1.4 meq. per minute, respectively. It can be seen that the patients excreted sodium and chloride at rates of two to two and one-half times greater than normal over a wide range of solute loads.

Renal Osmotic Work.—Renal osmotic work calculated from the formula of von Rhorer[23] takes into account the resultant energy in passing from state A,

plasma, to state B, urine. In the classical thermodynamic sense, the energy, thus calculated, depends only on the initial and final states, and is independent of the path or paths followed in going from plasma to urine. Furthermore, the amount of energy ascribed to a given state of any system is determined by the state itself, and not by its previous history.[24] It follows that calculated values of renal osmotic work describe only the minimum free energy expanded by the kidney during the production of a finite volume of urine from an infinite volume of plasma. Such values do not describe the cardiac source of filtration energy, intricate tubular mechanisms such as stepwise reabsorption and secretion of solutes, or enzymatic activity.

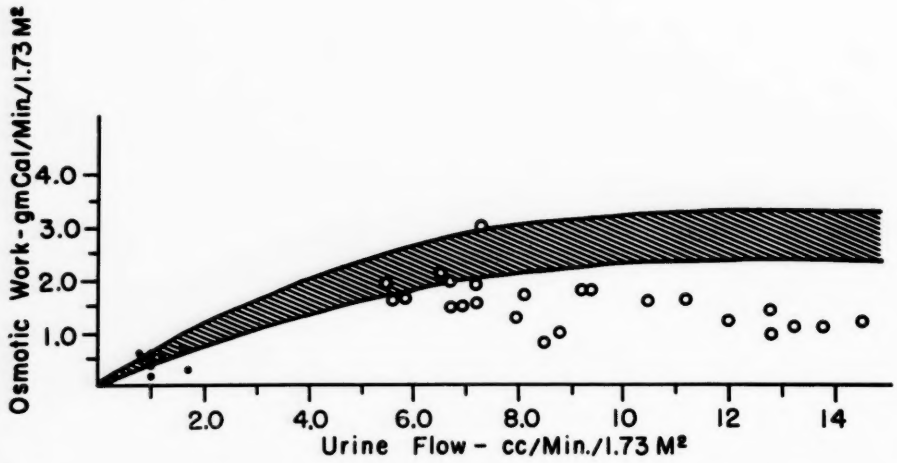

Fig. 5.—Thermodynamic work versus urine flow in hypertensive patients. Shaded area equals range of normal values.

It has long been known that osmotic work is but a small fraction of the total energy consumption of the kidney.[23, 25, 26, 27, 28] However, previous reports have shown that constant and reproducible values were obtained under conditions of osmotic loading. Despite the numerous limitations of a hypothetical nature, it seemed possible that such calculations could have biologic significance, in so far as they provide a quantitation of the minimum free energy expenditure of the kidney in the elaboration of urine from plasma. That the energy cost of filtration accounts for but a small fraction of even the renal osmotic work was discussed elsewhere.[20]

Fig. 5 presents, for 34 collection periods, 24 of which were collected during loading, a graph of the values of ideal osmotic work versus urine flow. It can be seen that data obtained from hypertensive subjects fall significantly below the normal range. At a urine flow of 14 ml. per minute, calculated work amounted to but 1.2 gram calories per minute. At similar flows in normal subjects, work values of approximately 3 gram calories were found.[20] The over-all pattern of work versus flow is suggestive of the existence of a reduced ceiling for osmotic work in the hypertensive kidney.

Clearances.—Table IV presents the mean values for clearances of urea, mannitol, and PAH for the six patients during loading periods. Each value represents the average of three to five clearance periods. In patients J.W., C.W., L.B., and S.S., the average value of clearance of urea was 54.0 to 57.6 ml. per minute, for that of mannitol 63.5 to 68.7, and for PAH, 312 to 441. These values were significantly less than those found in normal hydropenic subjects under identical loading conditions.[20] Calculated filtration fractions of 0.18 to 0.20 in 4 patients, higher than those of normal subjects, are in accord with data of others who have studied clearances of hypertensive patients.[7, 13] Patients E.K. and S.T. had normal hydropenic levels of mannitol and urea clearances and high values for filtration fraction.

Patient E.K. presented findings worthy of supplementary comment. She responded in a normal manner to mannitol loading in so far as relationships among values of urinary osmotic activity, flow, and load were concerned. The value of mannitol clearance was the same as, while that of PAH was significantly lower than, the mean value of 41 periods of loading in 16 normal hydropenic subjects. The rate of salt loss observed was almost twice the maximum rate observed in normal subjects under the same dietary, hydropenic, and loading conditions. The patient was 17 years of age, the youngest of the group, and known to be hypertensive (170/124) for but one year. She was completely asymptomatic and complained of no symptoms referable to cardiac, renal, or cerebral systems. Other than hypertension, no signs of cardiovascular disease were found. It would appear that the earliest renal manifestations of hypertensive disease in E.K. were a reduction in renal blood flow together with an excessive rate of salt excretion.

TABLE IV. VALUES FOR CLEARANCES DURING OSMOTIC DIURESIS IN HYPERTENSIVE PATIENTS

SUBJECT	CLEARANCES (POST-LOADING) UREA	MANNITOL C.C./MIN./1.73 M²	PAH	FILTRATION FRACTION	Cu./Cm.
J. W.	54.0	63.5	312	0.20	0.85
C. W.	57.6	66.2	447	0.15	0.87
E. K.	54.7	80.9	441	0.18	0.68
L. B.	60.3	68.7	538	0.13	0.88
S. S.	46.2	65.0	319	0.20	0.71
S. T.	56.6	85.5	478	0.18	0.65
Mean values*	63.3±1.8	83.6±2.1	596±16	0.14±0.002	0.75±0.01

*Mean values with their standard errors for clearances obtained during 41 periods of urine collection in 16 normal subjects under conditions of osmotic diuresis with mannitol loading.[17]

DISCUSSION

Previous papers on osmotic diuresis[19, 20, 21, 28] have yielded data on the composition of urinary solutes together with those of flow-load relationship, from which one, by inference, may localize the site of renal tubular defects. Thus, an abnormal composition of urinary solutes with a normal flow-load pattern suggests a tubular defect proximal to the site of concentration of the urine.[20, 29, 30] Conversely, normal composition of solutes with an abnormal flow-load pattern

implies a distal tubular defect.[31] Data of the present report indicate that hypertensive patients, under standard conditions of mannitol diuresis, excrete a urine wherein both factors, composition of solutes and flow-load relationship, are abnormal. Therefore, one may postulate the presence of functional defects at both proximal and distal loci of the nephron.

A salt-losing state during hydropenia and osmotic stress may be inferred from the observed abnormality of composition of urinary solutes, i.e., from the high rates of Na and Cl loss per unit of total solute excreted. From previous considerations,[20, 29, 30] the abnormal composition of urinary solutes may be attributed to renal dysfunction at a site in the tubule proximal to that of concentration or dilution of the urine.

Failure to correlate the rate of sodium excretion with that of glomerular filtration is apparent from the depressed values for clearances of mannitol during periods of excessive Na and Cl loss. This finding suggests that during the osmotic diuresis of hypertensive, as well as that of normal subjects, sodium excretion is predominantly a function of tubular activity.

The mechanism whereby tubular dysfunction, together with lowered rates of glomerular filtration and renal blood flow, results in an excessive excretion of salt, cannot be defined clearly from data of the present paper. Analogous changes of urinary function under different conditions have been observed by other workers. Thus, Verney[32, 33] noted that a primary reduction of renal blood flow (ligation of a major branch of one renal artery) in the dog produced increased excretion of chlorides and water on the operated side, confirming the work of Bradford,[34, 35] who had reported a similar effect after excision of a wedge of kidney substance. Such data suggest that a primary reduction in renal blood flow may produce changes in tubular activity with respect to transfers of water and solutes.

Whether such changes of tubular activity are due to hypoxia of tubular cells or to increased intrarenal and peritubular pressure per se cannot be decided at present. Evidence has been adduced in favor of either factor provoking changes in the excretion of water and salt. Thus, anoxic anoxia of both man and animals[38, 39] has been shown to result in natruria and polyuria without apparent change in renal hemodynamics. The role of hemodynamic factors has been established by Richards and Plant,[36] who demonstrated that increases of renal arterial pressure caused almost parallel increases in urine flow, and chloride excretion, while renal blood flow remained constant. More recently, their work has been confirmed by Shipley and Study,[37] who concluded that the increasing urine flow following mechanical increase of renal artery pressure in dogs is caused by increased intravascular and renal tissue pressure, which, in turn, is responsible for diminished transfer of filtrate through the tubular wall.

The polyuria and natruria observed in our patients occurred concomitantly with normal or reduced rates of filtration and renal blood flow as well as with high arterial blood pressure. Such observations may be ascribed equally well to factors of either increased intrarenal pressure or hypoxia

acting on tubular mechanisms for transport of solutes and water. Similar findings of polyuria and natruria have been noted in rabbits or rats made hypertensive by repeated injections of renin[40-42] and in essential hypertension of man during water diuresis.[4] In this connection, Weston and his colleagues[48] report finding an "electrolyte leak" in patients with hypertension.

Several workers have found evidence of salt retention in various stages of hypertensive disease.[1, 43, 47] However, our data as well as that of others[4, 37, 40, 42] indicate that under certain conditions salt-losing may be a common concomitant finding in apparently uncomplicated hypertensive disease.

SUMMARY

Six patients with uncomplicated hypertensive disease, under standard conditions of hydropenia, were tested by the technique of osmotic loading. Mannitol as the loading solute was given in amounts sufficient to add 20 milliosmoles per liter of distribution volume.

Urinary flow was higher and osmotic activity lower than normal under both "resting" or preliminary and loading conditions. Subjects were excreting urine at rates of 0.33 to 1.70 ml. per minute, and submaximal osmolarities of 557 to 806 milliosmoles per liter. After loading, flows rose to 10 to 14 ml. per minute, and osmolarity fell to 375 to 400. Data on the flow-load relationship showed that patients with hypertension required one and one-half times the normal urinary volume for excretion of a given solute load.

Minute losses of Na and Cl during diuresis were two to two and one-half times greater than normal, reaching values of 1.8 and 1.4 meq. per minute, respectively. The per cent composition of urinary solutes differed from that of normal subjects under identical loading conditions. While Na and Cl comprised higher, mannitol and urea comprised lower than normal proportions of the total urinary solutes. Calculated values of ideal osmotic work amounted to less than one-half those of normal subjects.

Clearances of mannitol, at 63.5 ml. per minute, were 25 per cent below normal, while those of PAH, at 312 ml., were 50 per cent of normal. Filtration fractions were elevated.

The significance of concentration defects and excessive excretion rates of salt together with reduced clearances of mannitol and PAH was discussed. It was postulated that defective concentrating ability implied distal, and distorted composition of urinary solutes, proximal tubular dysfunction.

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THE USE OF COBALTOUS CHLORIDE IN THE ANEMIA ASSOCIATED WITH CHRONIC RENAL DISEASE

FRANK H. GARDNER,* M.D.
BOSTON, MASS.

INTRODUCTION

HEMATOPOIETIC responses to orally administered cobaltous chloride have been reported by Berk and associates[1] in patients with a variety of diseases as well as in normal subjects. Robinson and associates[2] showed that the oral administration of cobalt would improve the mild anemias associated with chronic suppurative infections. This report appeared to confirm the animal studies with turpentine abscesses done by Wintrobe and his colleagues.[3] The recent studies, as well as the clinical experiences of Weissbecker,[4] suggest that cobalt functions as a nonspecific erythropoietic stimulant rather than as a specific nutritional component required for red cell or hemoglobin production.

Past studies have emphasized the progressive anemia usually associated with chronic azotemia[5] and the rough correlation between degree of nitrogen retention and hemoglobin depression. No adequate explanation has been offered for these observations. The association of chronic azotemia and anemia, therefore, can be accepted as a clinical observation without physiological understanding at this time. Although most investigators have classified renal anemia as due to toxic inhibition of erythropoiesis, the chemical abnormalities that have been measured in renal insufficiency are not known to be specific bone marrow inhibitors. Loge and associates[6] and Emerson and Burrow[7] have suggested that the anemia may in some cases have a hemolytic factor, as evaluated by transfused red cell survival studies. Observation in this clinic would suggest that most patients have an anemia because of bone marrow inhibition, although a few have an associated hemolytic factor.[8] Additional observation will be published at a later date concerning these laboratory findings.

The past therapy in this medical clinic for the anemia associated with azotemia has been repeated transfusions. However, in many patients repeated transfusions are associated with increasing numbers of reactions. The majority of these reactions have not been hemolytic, but of a nonspecific febrile type associated with nausea and vomiting. Fever and anorexia from these reactions may only add to the burden of the embarrassed kidney function. Because of the recent experience with cobaltous chloride as an erythropoietic agent, a program was initiated with the oral use of this metal in renal anemia. Efforts were directed to (1) eliminate repeated blood transfusions, (2) determine whether

From the Medical Clinic, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School.

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*Associate, Harvard Medical School; Associate in Medicine, Peter Bent Brigham Hospital.

patients felt subjectively improved with consistently higher hemoglobin levels, (3) evaluate the toxicity of this drug upon prolonged administration.

METHODS AND MATERIALS

Complete blood values including reticulocyte counts and differential counts of the peripheral blood film were calculated from venous blood samples rendered incoagulable by balanced oxalate.[9] An aspiration of bone marrow from the iliac crest or sternum was done in the majority of patients to help exclude bone marrow involvement by other causes. Slide and cover glass smears of marrow particles were prepared and stained with Wright's stain. No quantitative enumeration of the individual cellular elements was made from these smears for this study, although they were evaluated for an estimation of cellularity and relative number of erythrocytic and granulocytic precursors. In five patients the percentage of erythroid precursors in 500 nucleated cells was determined before and during the cobalt therapy. Total blood volumes and red cell volumes were determined with Evans Blue dye[10] before and during therapy in nine of the patients.*

The patients used in this study were referred by the hospital staff.† The classification of renal disease by the cardiorenal clinic has been accepted in this study. Patients were selected, for the most part, because of marked blood urea nitrogen retention and anemia. Although the majority of patients had blood values tabulated for several months while they were under study in the hospital or clinic, all patients had at least two complete blood studies done before cobalt therapy was initiated. No effort was made to alter supportive therapy that was deemed necessary for improving the electrolyte imbalance complicating azotemia. This supportive therapy in these patients as well as others in the clinic has not altered the anemia associated with uremia.

Patients received cobaltous chloride orally.‡ In the early phase of this study a 50 mg. uncoated tablet was used. However, because of gastrointestinal distress, a 20 mg. enteric coated tablet was prepared. Each patient was given 50 to 150 mg. total daily dose and instructed to take the medication with meals. The patients were observed carefully at the onset of therapy to prevent any gastrointestinal irritation that might result in vomiting and the added burden of extra renal azotemia. The dosage was increased at weekly intervals to the patient's tolerance or to the level where a hematopoietic response was apparent.

RESULTS

Seventeen patients were studied for at least a four-week period. In 18 additional patients the rapid decline in health with progression of azotemia and acidosis did not allow adequate evaluation or continuation of cobalt therapy. Early in the study it became evident that no significant erythropoietic response could be elicited in less than a four-week period of cobalt therapy, and therefore no patients are included who received less than one month of therapy. The observations on the 17 patients studied for four or more weeks will be reported in this study.

There were no essential changes in the white blood counts, differential counts of peripheral blood film, or platelets in the group of patients studied. The blood values before and during cobalt therapy are listed in Table I. The majority of patients with this type of anemia manifest a variable reticulocytosis.

*Because of lipemia in Case 15, blood volume studies were done with radioactive chromium through the courtesy of Dr. S. Gray and Dr. K. Sterling.[21]

†Dr. George W. Thorn, Dr. John P. Merrill, and Dr. James P. O'Hare referred the majority of patients in this study. The author is indebted to them for their cooperation and kindness in allowing study of these patients. Hematologic studies of Case 13 were done by Dr. Ralph Wallerstein, Thorndike Memorial Laboratory, Boston City Hospital.

‡The author is indebted to Smith, Kline and French Laboratories, Philadephia, for preparation of these tablets.

TABLE I. HEMATOLOGIC CHANGES PRODUCED BY COBALTOUS CHLORIDE ($CoCl_2$) THERAPY

PATIENT	DIAGNOSIS	B.U.N.† (RANGE)	$CoCl_2$* (MG./DAY)	DURATION OF THERAPY (WEEKS)	R.B.C. X10⁶/MM.³		HEMOGLOBIN (GM./100 ML.)		HEMATOCRIT (PER CENT)		RED CELL VOLUME (ML.)	
					BEFORE	DURING	BEFORE	DURING	BEFORE	DURING	BEFORE	DURING
1. F. W.	Chronic pyelonephritis	90-100	100	14	3.11	5.01	9.4	12.4	30	40	501	990
2. A. C.	Chronic pyelonephritis	60- 80	100	8	2.75	3.24	8.04	10.5	25	33	1,150	1,290
3. P. S.	Chronic pyelonephritis	80-110	*80*	10	3.1	4.05	8.5	12.3	26	38	—	—
4. C. S.	Chronic pyelonephritis	60- 75	*100*	28	3.1	4.52	11.0	12.8	33	38	1,577	1,782
5. W. L.	Chronic pyelonephritis	50- 70	*80*	16	3.2	4.0	10.0	11.7	31	36	1,312	1,462
	(second study)	50- 70	*100*	8	3.2	4.1	9.2	11.7	28	38	—	—
6. J. C.	Chronic pyelonephritis	80- 90	*100*	16	2.7	4.8	8.7	12.9	26	41	990	1,395
7. R. A.	Chronic pyelonephritis	20- 40	*100*	4	3.4	3.6	10.6	12.3	32	38	—	—
8. J. G.	Chronic pyelonephritis	100-110	100-200	20	3.0	4.7	8.8	11.8	26	39	—	—
9. E. M.	Chronic glomerulonephritis	60- 90	100	14	2.9	4.49	9.1	13.1	26	40	1,272	1,680
10. D. P.	Chronic glomerulonephritis	80-114	100-150	44	2.95	4.8	6.0	9.6	17	31	—	—
11. C. S.	Chronic glomerulonephritis	70- 80	*100*	14	3.15	3.85	10.6	11.8	31	38	1,423	1,773
12. D. B.	Chronic glomerulonephritis	70- 90	150	8	3.51	4.1	10.2	12.1	29	40	992	1,432
13. W. P.	Chronic glomerulonephritis	80-100	100	7	2.8	4.14	7.6	10.3	24	32	—	—
14. D. H.	Chronic glomerulonephritis	70- 90	100	12	3.7	4.15	11.0	13.1	29	38	—	—
15. K. M.	Nephrotic syndrome	15- 20	*80*	10	2.8	5.02	9.1	14.6	28	45	775	1,620
16. J. V.	Acute glomerulonephritis	11- 15	50	8	3.92	5.05	9.8	14.2	29	43	—	—
17. B. B.	Polycystic kidney disease	80	50	12	3.4	—	8.6	11.4	28	37	—	—

*Italicized underlined dosage schedule represents enteric coated tablets.

†B.U.N. = blood urea nitrogen in milligrams per cent.

During the control periods of observation the percentage of reticulocytes ranged from 1 to 4 per cent. During periods of cobalt therapy there was no consistent increase in the reticulocyte percentage that could be specifically attributed to the therapy, for the same variability was present as existed during the pretreatment period. Therefore, reticulocyte values are not tabulated. Reticulocyte responses cannot be used to evaluate the erythropoietic response to cobalt in this type of anemia. The red blood counts, hemoglobin values, and hematocrit percentages in Table I represent average control values before therapy and the maximum response during therapy. Most patients showed a significant erythropoietic response in one month, and maximal values were reached in a two- to

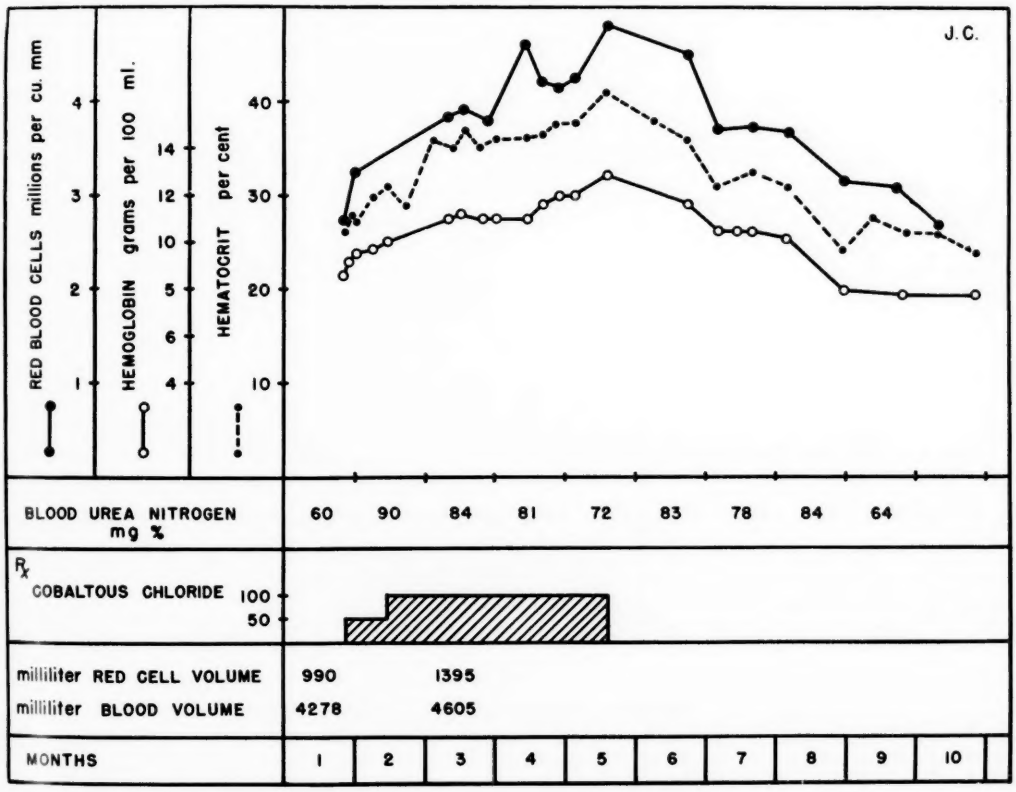

Fig. 1.—Case 6 (J. C.), chronic pyleonephritis.

three-month period. In some patients therapy was continued for many weeks to evaluate toxicity of the drug. Cases 6, 9, and 10, as illustrative results, are charted in Figs. 1, 2, and 3. All cases except 8, 11, and 14 were followed for a twelve-week period after cobalt therapy was discontinued. During this twelve-week period all patients had a decline to pretreatment levels in blood values comparable to those shown in Figs. 1 to 3. The peripheral blood films of all patients revealed a normocytic, normochromic type of anemia except Case 10. It was believed that this patient had thalassemia minor (Cooley's trait) on the

basis of racial and family history, although a family study was not possible. The peripheral blood film had the marked variation in size and shape of the red cells associated with hypochromia and target forms to substantiate the diagnosis. Although the patient experienced an erythroid response to cobalt therapy, the red cells remained hypochromic and microcytic. Case 16 with acute glomerulonephritis showed normal blood values after eight weeks of therapy. Again, when cobalt was withheld the hemoglobin dropped to 11 Gm., and it is assumed that the patient has subacute glomerulonephritis.

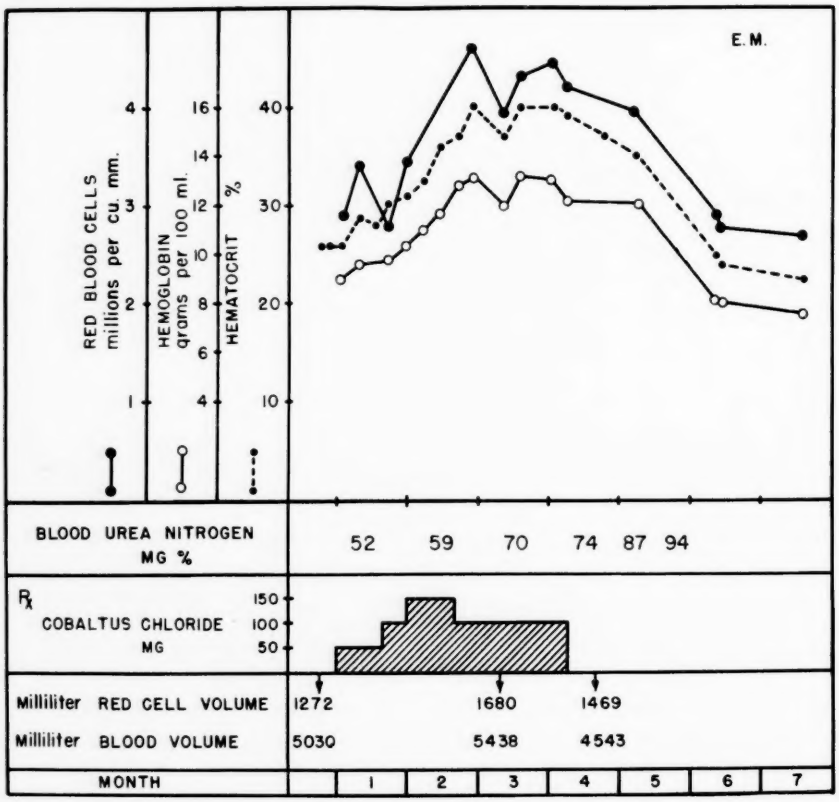
E. M.
RED BLOOD CELLS millions per cu. mm.
HEMOGLOBIN grams per 100 ml.
HEMATOCRIT %
BLOOD UREA NITROGEN MG %
52 59 70 74 87 94
Rx COBALTUS CHLORIDE MG
Milliliter RED CELL VOLUME
1272 1680 1469
Milliliter BLOOD VOLUME
5030 5438 4543
MONTH
1 2 3 4 5 6 7

Fig. 2.—Case 9 (E. M.), chronic glomerulonephritis. The blood values decline in a two-month period after cobalt therapy is discontinued.

The indirect measurements of red cell volume with Evans Blue done in this study do not correlate necessarily with the other peripheral blood measurements. These values are tabulated in Table I to demonstrate that in most cases the increase in red cell mass only approximates the changes in red blood count, hemoglobin, and hematocrit. Evans Blue is carried in the plasma bound to albumin and some of the dye is lost with such protein in the urine.[11] The presence of edema or increased capillary permeability in nephritis allows increased loss of the dye during the test period.[12] If possible, red cell volume should be measured by a method that tags the erythrocyte as done in Case 15. Such a technique was not available for this study.

Five patients had bone marrow aspirations done before and at the time of maximal response to cobalt therapy. As noted in Table II, four of the patients had a distinct increase in the percentage of erythroid precursors. The significance of this percentage increase cannot be evaluated in this limited study. However, Kato has previously reported erythroid hyperplasia of the bone marrow in animals receiving cobalt.[13]

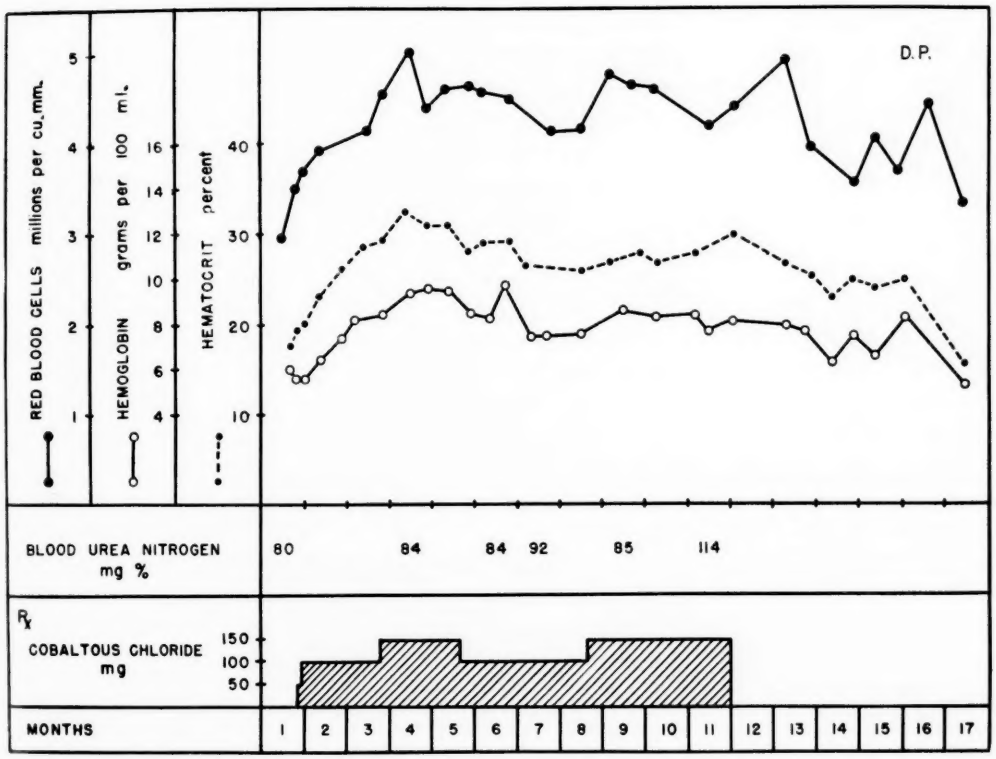

Fig. 3.—Case 10 (D. P.), thalassemia minor (Cooley's trait) associated with chronic glomerulonephritis.

TOXICITY

It has been emphasized previously that oral ingestion of cobalt can cause nausea and vomiting.[1] This complication is more prone to exist if the drug is given in liquid form.[1] In the cases reported here a dosage schedule in excess of 100 mg. daily usually caused anorexia, and eventually nausea and vomiting. Most patients have had no gastrointestinal symptoms at a dosage schedule of 100 mg. when enteric coated tablets were used and the medication taken during meals. One patient complained persistently of loose stools during the entire period of therapy, but none had diarrhea. Four patients noted tinnitus after four to sixteen weeks of therapy. This complaint was volunteered by one patient and elicited in three others by questioning. One of this group (Case 5) had onset of nerve deafness after twelve weeks of therapy. This deafness was severe and hearing was absent in a frequency range above 1,000 cycles per second. Full

TABLE II. PERCENTAGE OF BONE MARROW ERYTHROID PRECURSORS BEFORE AND DURING COBALT THERAPY

CASE	BEFORE	DURING
Case 1 (F. W.)	12.2	18.0
Case 5 (W. L.)	11.2	14.0
Case 6 (J. C.)	12.1	21.6
Case 9 (E. M.)	14.0	20.4
Case 15 (K. MacC.)	16.6	27.4

recovery of hearing was obtained over the ten-week period after the drug was discontinued and the audiogram charts returned to the same range of hearing as was observed before therapy. Inasmuch as this patient had some defect in hearing before the study was initiated, some doubt existed as to the etiology of the transient deafness. A second period of therapy was begun, and nerve deafness again occurred after eight weeks of treatment. The patient again has had a good recovery of function when the drug was discontinued.*

DISCUSSION

These studies show that oral cobalt therapy can stimulate erythropoiesis and relieve the anemia in patients with chronic renal disease. None of the patients in this study had been treated previously with frequent transfusions. No observations were made to demonstrate that these patients had fewer hospital admissions. However, the increased erythropoietic activity would preclude the need for transfusions. Patients had improved appetites and increased tolerances for the medications necessary to correct electrolyte abnormalities. One patient (Case 1) had longer intervals between extrarenal dialysis[14] when hemoglobin was elevated. Finally, the toleration of electrolyte imbalance was greater with increased hemoglobin values. The general expression of improved well-being and improvement by the patient is difficult to evaluate. Most patients were seen at weekly intervals early in the study. Patients had the opportunity to express complaints and receive assurance. Minor changes in therapeutic programs were made frequently to give the patient comfort.

Because of the marked impairment in renal function, no laboratory studies were done to evaluate progression of disease aside from frequent determinations of blood urea nitrogen and necessary procedures to evaluate and control acidosis. No evidence was obtained to suggest that cobalt therapy had altered the course of the disease.† Past literature has emphasized the nephrotoxic activity of cobalt in animals.[15, 16] In those studies the amounts of cobalt given were far in excess of the oral dosages used in this group of patients. Previous studies suggest that 6 to 9 per cent of orally ingested cobalt is eliminated by the kidney in normal individuals.[4] At the present time one cannot state whether the renal excretion or retention of this metal is a toxic hazard to the patient. Studies

*One patient, not included in Table I, who received 400 mg. of uncoated tablets of cobaltous chloride daily for a six-week period, complained of paresthesias in his feet. These paresthesias were described as a burning sensation on the soles with numbness of the toes. At this time vibration sensation was absent in the toes although no observation of this had been recorded prior to therapy. These symptoms disappeared and vibration sensation returned when therapy was discontinued.

†Case 10 received the drug for forty-four weeks without essential change in the course of the disease. Case 5 has been followed for one year after two periods of therapy without marked alteration in the progress of pyelonephritis.

are now in progress to evaluate the effect of cobalt in rats that have developed a nephrotoxic nephritis. Berk and associates[1] and other investigators[17] have reported that some patients have complained of substernal pain while receiving cobalt. This type of pain disappeared when the drug was discontinued. No patients had evidence of myocardial disease at the time pain was present or subsequently. In this clinic that complaint was not elicited, nor did the patients have any clinical evidence of change in the cardiovascular status while receiving cobalt. Likewise in the large clinical experience reported by Weissbecker,[4] there was no mention of chest pain or any neurological complications. Nevertheless, in view of the suggestive toxicity for eighth nerve involvement observed in this study in four patients, it is conceivable that one may foresee other toxic neurological properties appear with continued use of the drug.

Recently a parenteral cobalt amino acid complex has been studied.[18] It was given intramuscularly daily for periods of several weeks. Each daily injection contained 3 to 5 mg. of metallic cobalt. This preparation has the advantage of a constant slow release of the cobalt ion into the tissues because of limited dissociation of the complex. It is claimed that there is no gastrointestinal irritation from this preparation. However, there has been no adequate experience with this particular compound in this country.

Although this study does demonstrate that the anemia of uremia can respond to cobalt therapy, it does not elucidate the cause of the anemia or the effect of cobalt on the bone marrow. There is some evidence that cobalt may inhibit sulfhydryl groups in enzyme systems.[19] Cobalt polycythemia in animals can be inhibited by amino acids with sulfur components.[20] Such a mechanism suggests that the erythroid hyperplasia seen in the bone marrow after cobalt therapy may be associated with anoxia from interference of enzyme systems. At the present time this metal is the only agent that this clinic has found to produce an erythropoietic response in uremic patients. It should only be used with full understanding of the benefits in contrast to the toxic complications discussed. The direct effect on the kidney parenchyma from prolonged exposure to this metal in low concentration awaits further study.

SUMMARY

Seventeen patients with anemia associated with renal disease were treated four or more weeks with oral cobaltous chloride. During the period of treatment increased erythropoiesis was observed. No significant changes were noted in other circulatory blood elements. The increased blood values declined to pretreatment levels when the drug was discontinued. No mechanism is known to explain the erythropoiesis associated with cobalt therapy. The drug should be used with full recognition of the possible toxic complications.

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MAINTENANCE THERAPY OF PERNICIOUS ANEMIA WITH VITAMIN B_{12}

GORDON C. MEACHAM, M.D., AND ROBERT W. HEINLE, M.D.
CLEVELAND, OHIO

IT HAS been demonstrated adequately that vitamin B_{12} is effective in producing a satisfactory hematologic and clinical response in patients with pernicious anemia in relapse, and that it is effective in the therapy of neurological and lingual complications.[1, 2] The purpose of the present study was to determine whether vitamin B_{12} given parenterally over a long period of time is capable of maintaining complete remission in patients with pernicious anemia and, if so, to ascertain the dosage requirement necessary. In a previous report[3] it was shown that a vitamin B_{12} concentrate in a dosage averaging 1 μg daily given intramuscularly at intervals of three or four weeks failed to maintain optimal blood levels, although there was no development or progression of neural lesions in any case. It was concluded that the most likely explanation for this failure of adequate hematopoiesis was inadequate dosage, although other possibilities were considered.

MATERIALS AND METHODS

Forty-three patients with Addisonian pernicious anemia, proved by the presence of macrocytic anemia, megaloblastic bone marrow, histamine-fast achlorhydria, and in most cases a previous response to either liver extract or folic acid, are included in this study. Many had had lingual or neurological involvement at some time previously. Of this group, 21 patients previously treated at intervals of three or four weeks with vitamin B_{12} concentrate in a dosage averaging 1 μg per day for periods varying from two to fourteen months were treated with an increased dose of 3 μg daily of the concentrate (Normocytin, Lederle).* Nine patients previously treated with 1 μg daily of vitamin B_{12} concentrate for at least eleven months were started on 1 μg daily of crystalline vitamin B_{12} (Betalin 12, Lilly).† Thirteen patients were started initially on either 3 μg daily of the concentrate or 1 μg daily of crystalline vitamin B_{12}. The concentrate (Normocytin, Lederle) was a highly purified, although not crystalline, material supplied as a clear, pink solution containing 30 μg of vitamin B_{12} activity per milliliter, when assayed with *Lactobacillus leichmanii* 313. It was produced by the microorganism *Streptomyces aureofaciens* and was prepared by concentration of the pink fraction from the organism.[4]

RESULTS

In comparing the results of therapy with vitamin B_{12} concentrate and crystalline vitamin B_{12} arbitrary standards similar to those described previously[3] have been used again. A change in the erythrocyte count of 300,000 or less per

From the Department of Medicine, Western Reserve University School of Medicine, and University Hospitals.

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†Generously supplied by Eli Lilly & Company, Indianapolis, Ind.

TABLE I. RESULT OF ADMINISTERING INCREASED DOSE OF B_{12} CONCENTRATE (NORMOCYTIN, LEDERLE) AFTER PREVIOUS TREATMENT WITH SMALLER DOSE

PT. NO.	1ST COURSE NORMOCYTIN (MO.)	DOSE (μG.)	DOSE INTERVAL (WK.)	AVERAGE AFTER 1ST COURSE NORMOCYTIN		2ND COURSE NORMOCYTIN (MO.)	DOSE (μG.)	DOSE INTERVAL (WK.)	AVERAGE AFTER 2ND COURSE NORMOCYTIN		DIFFERENCE 1ST *vs.* 2ND COURSE		M.C.V. AT END OF 2ND COURSE
				R.B.C. (× 10^6)	HGB. (GM.)				R.B.C. (× 10^6)	HGB. (GM.)	R.B.C. (× 10^6)	HGB. (GM.)	
1	7	40	4	3.95	13.2	16	90	4	4.39	14.1	+0.44	+0.9	93.4
2	13	40	4	4.24	12.9	17	90	4	4.57	13.9	+0.33	+1.0	93.0
3	6	20	3	3.84	12.8	12	60	3	4.20	12.9	+0.36	+0.1	102.4
4	7	20	3	3.78	13.3	16	60	3	4.36	12.6	+0.58	−0.7	98.6
5	13	20	3	3.29	12.2	15	60	3	4.12	12.3	+0.88	+0.1	98.3
6	10	20	3	4.28	14.2	11	60	3	4.69	14.6	+0.41	+0.4	102.3
7	12	20	3	4.96	13.1	11	60	3	5.11	14.2	+0.15	+1.1	91.9
8	12	20	3	4.09	12.9	16	60	3	5.36	15.8	+1.27	+2.9	96.1
9	12	20	3	3.37	12.2	16	60	3	4.38	13.1	+1.01	+0.9	100.5
10	2	20	3	3.85	12.9	16	60	3	4.45	13.2	+0.60	+0.3	94.3
11	13	20	3	4.01	12.2	16	60	3	4.77	14.2	+0.76	+2.0	95.3
12	7	20	3	3.92	13.9	11	60	3	4.50	14.5	+0.58	+0.6	101.1
13	8	20	3	3.81	12.7	11	60	3	4.80	14.5	+0.99	+1.8	96.9
14	12*	20	3	3.67	12.7	17*	60	3	4.41	12.9	+0.74	+0.2	97.5
15	5	40-60	4	4.13	13.9	17	90	4	5.26	14.6	+1.13	+0.7	87.4
16	14	20	3	3.72	12.8	16	60	3	4.10	13.1	+0.38	+0.3	109.7
17	7	20	3-4	4.10	13.1	16	60	3	4.15	12.9	+0.05	−0.2	101.2
18	13	20	3	3.85	12.8	16	60	3	4.08	13.1	+0.23	+0.3	105.3
19	3	20	3	3.26	12.4	16	60	3	4.39	13.7	+1.13	+1.3	97.9
20	7	40-60	4	3.96	13.3	14	90	4	4.58	15.0	+0.62	+1.7	102.6
21	12	20	3	3.60	12.3	16	60	3	4.63	13.4	+1.03	+1.1	95.0

*Three-month lapse in therapy.

TABLE II. RESULT OF CHANGING FROM TREATMENT WITH B_{12} CONCENTRATE TO CRYSTALLINE B_{12}

PT. NO.	COURSE OF NORMOCYTIN (MO.)	DOSE (μG)	DOSE INTERVAL (WK.)	AVERAGE AFTER COURSE OF NORMOCYTIN		COURSE OF CRYST. B_{12} (MO.)	DOSE (μG)	DOSE INTERVAL (WK.)	AVERAGE AFTER CRYST. B_{12}		DIFFERENCE CRYST. *vs.* CONC.		M.C.V. AT END COURSE OF B_{12}
				R.B.C. ($\times 10^6$)	HGB. (GM.)				R.B.C. ($\times 10^6$)	HGB. (GM.)	R.B.C. ($\times 10^6$)	HGB. (GM.)	
22	14	20	3	3.71	12.5	16	22.5	3	4.76	14.0	+1.05	+1.5	91.4
23	12	20	3	3.50	12.3	16	22.5	3	3.91	12.4	+0.41	+0.1	106.1
24	11	20	3	3.91	12.9	10	22.5	3	4.45	13.0	+0.54	+0.1	100.0
25	14	20	3	3.82	13.0	16	22.5	3	4.32	12.8	+0.50	−0.2	101.8
26	12	20	3	3.57	11.6	18	22.5	3	4.02	11.2	+0.45	−0.4	90.7
27	12	20	3	2.96	10.5	11	22.5	3	3.39	10.8	+0.43	+0.3	109.1
28	13	20	3	4.65	15.5	11	22.5	3	4.59	14.1	−0.06	−1.4	103.5
29	14	20	3	3.89	13.3	16	22.5	3	4.23	13.9	+0.34	+0.6	105.2
30	13	20	3	3.87	13.4	7	22.5	3	4.24	13.8	+0.37	+0.4	106.1

cubic millimeter is not considered significant. A change of 300,000 to 500,000 is considered moderate, and a change of over 500,000 is considered marked. All hematologic determinations were made by the same two technicians using uniform equipment. Erythrocyte and hemoglobin determinations were made on each visit, and hematocrit determinations were made at appropriate intervals.

Twenty-one patients were treated with the increased dosage of vitamin B_{12} concentrate (average 3 μg per day at intervals of three or four weeks) for at least eleven months, and in fourteen instances for sixteen months or longer (Table I). In eighteen patients there was a significant improvement in the erythrocyte level. In five, there was an increase of over 1,000,000 cells per cubic millimeter. In eight others, there was an increase of over 500,000 cells per cubic millimeter. Five patients had an increase of 300,000 to 500,000 cells per cubic millimeter, and three showed an increase of less than 300,000 cells. In no instance was there a decrease in the erythrocyte level. Whereas at the beginning of this period of treatment, fourteen of the twenty-one patients had erythrocyte levels less than 4,000,000 cells per cubic millimeter, at the end of treatment with the increased dosage all of the patients had higher levels (Table I).

Nine patients, who had received an average of 1 μg daily of the vitamin B_{12} concentrate for a period of at least eleven months, were changed to an average daily dose of 1 μg of crystalline vitamin B_{12} (Betalin 12, Lilly) and followed from seven to eighteen months (Table II). At the beginning of treatment with this material eight of the nine had erythrocyte levels less than 4,000,000 cells per cubic millimeter. In two patients, the erythrocytes increased more than 500,000 cells per cubic millimeter. In six, there was an increase of 300,000 to 500,000 cells per cubic millimeter. In the one patient (No. 28, Table II), who originally had a higher normal erythrocyte count, there was an insignificant decrease of 60,000 cells per cubic millimeter. At the end of this period of therapy, seven of the nine had erythrocyte levels between 4,020,000 and 4,760,000 cells per cubic millimeter. Two patients remained below 4,000,000, but both had higher levels than before this treatment period. One patient, (No. 27, Table II), an 89-year-old white man, has failed to maintain good levels for many years in spite of vigorous treatment with liver extract and folic acid. Repeated marrow aspirations have shown abnormally large amounts of fat, a condition unrelieved by the administration of choline and other adjunct therapy.

Eleven patients were placed on therapy with an average of 3 μg daily of vitamin B_{12} concentrate (Table III). Five of these, initially seen in relapse, were first treated with small oral doses of vitamin B_{12} and fractions of hog stomach mucosa in an experimental program with varying degrees of response. One patient (No. 35, Table III), was admitted in mild hematologic and neurologic relapse, and was treated vigorously with vitamin B_{12} while in the hospital. The others had received either crude liver extract (2 U.S.P. units per milliliter) or folic acid previously. In three patients erythrocytes increased from 300,000 to 500,000 cells per cubic millimeter, and five had an increase of over 1,000,000 cells. Three others showed a decrease of less than 300,000 cells. However, only one patient had an erythrocyte count of less than 4,000,000 cells per cubic

TABLE III. PATIENTS STARTED ON MAINTENANCE WITH LARGER DOSES OF B_{12} CONCENTRATE, AVERAGING 3 μG PER DAY

PT. NO.	R.B.C. AND HGB., START OF THERAPY		DURATION OF THERAPY (MO.)	DOSE (μG)	DOSE INTERVAL (WK.)	R.B.C. AND HGB. AT END OF THERAPY		DIFFERENCE IN R.B.C. AND HGB. START *vs.* COMPLETION		M.C.V. AT END OF COURSE OF NORMOCYTIN
	R.B.C. ($\times 10^6$)	HGB. (GM.)				R.B.C. ($\times 10^6$)	HGB. (GM.)	R.B.C. ($\times 10^6$)	HGB. (GM.)	
31	3.40	11.6	10	60	3	4.51	14.2	+1.11	+2.6	100.9
32*	4.41	12.9	16	60	3	4.38	13.8	−0.03	+0.9	100.9
33†	3.87	12.1	16	60	3	4.19	12.5	+0.32	+0.4	95.5
34	3.27	10.8	11	60	3	3.26	11.1	−0.01	+0.3	101.2
35	4.40	12.4	16	60	3	4.13	12.2	−0.27	−0.2	93.9
36	2.83	11.6	5	60	3	4.04	12.6	+1.21	+1.0	107.6
37*	3.37	12.7	3	60	1-3	4.76	14.7	+1.39	+2.0	102.9
38*	3.90	12.7	16	60	3	4.22	12.8	+0.32	+0.1	96.9
39	2.90	12.3	6	60	2-3	4.50	13.4	+1.60	+1.1	100.0
40†	4.25	13.3	16	90	4	4.62	14.5	+0.37	+1.2	89.7
41	2.66	11.7	8	60	2-3	4.36	13.3	+1.70	+1.6	94.0

*Previously treated with crude liver extract.
†Previously treated with folic acid.

TABLE IV. PATIENTS STARTED ON MAINTENANCE WITH CRYSTALLINE B_{12} AVERAGING 1 μG PER DAY

PT. NO.	R.B.C. AND HGB. AT START OF THERAPY		DURATION OF THERAPY	DOSE (μG)	DOSE INTERVAL (WK.)	R.B.C. AND HGB. AT END OF COURSE		DIFFERENCE IN R.B.C. AND HGB. START *vs.* COMPLETION		M.C.V. AT END OF COURSE
	R.B.C. ($\times 10^6$)	HGB. (GM.)				R.B.C. ($\times 10^6$)	HGB. (GM.)	R.B.C. ($\times 10^6$)	HGB. (GM.)	
42	3.52	11.7	10	22.5	3	4.25	14.4	+0.73	+2.7	112.9
43	2.96	8.8	7	22.5	3	4.51	13.3	+1.55	+4.5	86.0

TABLE V. EFFECT OF PURIFIED LIVER ON PATIENTS WITH MACROCYTOSIS

PT. NO.	PREVIOUS THERAPY	DURATION (MO.)	DOSE (μG)	DOSE INTERVAL (WK.)	VALUES AT START OF LIVER THERAPY			DURATION LIVER THERAPY (MO.)	DOSE PURIFIED LIVER (UNITS)	DOSE INTERVAL (WK.)	VALUES AT END OF LIVER THERAPY		
					R.B.C. ($\times 10^6$)	HGB. (GM.)	M.C.V.				R.B.C. ($\times 10^6$)	HGB. (GM.)	M.C.V.
31	Normocytin	10	60	3	4.51	14.2	100.9	10	30	4	4.65	14.0	94.7
3	Normocytin	12	60	3	4.20	12.9	102.4	4	30	4	4.53	13.9	100.4
36	Normocytin	5	60	3	4.04	12.6	110.7	4	30	4	3.93	12.9	110.7
6	Normocytin	11	60	3	4.69	14.6	102.3	4	30	4	4.76	15.0	102.9
12	Normocytin	11	60	3	4.50	14.5	101.1	4	30	4	4.58	13.9	100.4
39	Normocytin	6	60	2-3	4.50	13.4	100.0	4	30	4	4.05	13.5	109.8
24	Cryst. B_{12}	10	22.5	3	4.45	13.0	100.0	4	30	4	4.48	13.5	99.3
27	Cryst. B_{12}	11	22.5	3	3.39	10.8	109.1	4	30	4	3.62	11.1	100.8

millimeter at the end of treatment. This patient (No. 34, Table III), was a 77-year-old white man with severe cardiac decompensation, angina pectoris, and dorsolateral column degeneration. When initially seen in relapse, he was treated with small oral doses of vitamin B_{12} and fractions of hog stomach during an experimental program with a moderately good initial response.

Two patients seen in relapse and treated initially with small oral doses of vitamin B_{12} and hog stomach fractions were started on treatment with crystalline vitamin B_{12}, averaging 1 μg daily administered at intervals of three weeks. Both responded well with an increase of over 500,000 erythrocytes per cubic millimeter (Table IV).

After ten or more months of therapy with the larger dose of vitamin B_{12} concentrate or the smaller dose of crystalline vitamin B_{12}, it became apparent that, while all but three of the forty-three patients had attained and maintained satisfactory erythrocyte levels, macrocytosis (mean corpuscular volume greater than 100 cubic microns) was not an uncommon finding. Of the 43 patients treated, 22 had a mean corpuscular volume of 100 cubic microns or greater. Eight of the patients who were being treated with the increased dose of vitamin B_{12} concentrate or the smaller dose of crystalline B_{12} were changed to treatment with purified liver extract (Lederle, 15 U.S.P. units per milliliter) in an intramuscular dose of thirty units given at intervals of twenty-eight days (Table V). At the end of four or more months there was no significant lowering of the mean corpuscular volume in any instance, except that one patient (No. 31, Table V), treated for two months, had a decrease in the mean corpuscular volume from 100.9 cubic microns to 94.7 cubic microns. In six instances there was no significant change in the erythrocyte levels. One patient showed an increase of 330,000 cells, while another showed a decrease of 450,000 cells per cubic millimeter.

To ascertain the effect of folic acid on the macrocytosis, eighteen patients, in all of whom the mean corpuscular volume was greater than 100 cubic microns, were begun on folic acid in an oral dose of five mg. a day and were continued on the increased dosage of vitamin B_{12} concentrate, the smaller dose of crystalline vitamin B_{12}, or purified liver extract (Table VI). In only three instances did the mean corpuscular volume fall below 100 cubic microns after five to seven months of treatment, and in no case did it fall to or below 95 cubic microns. The average change was –1.2 cubic microns, certainly insignificant. Four patients showed a significant increase in erythrocyte levels, whereas two showed a significant decrease.

DISCUSSION

It is clear that, whereas the vitamin B_{12} concentrate used in the earlier experiments in doses averaging 1 μg daily was unable to maintain normal blood levels, as previously reported,[3] the larger dose averaging 3 μg daily caused improvement in erythrocyte levels in every instance. Therefore, an average of 1 μg daily of this material given intramuscularly at intervals of three or four weeks was not adequate therapy. With the larger dose, erythrocyte levels of 4,000,000 cells per cubic millimeter or more were achieved or maintained in all

TABLE VI. RESULTS OF ADMINISTERING FOLIC ACID, 5 MG. ORALLY DAILY, IN ADDITION TO TREATMENT EITHER WITH B_{12} CONCENTRATE, CRYSTALLINE B_{12}, OR PURIFIED LIVER EXTRACT

PT. NO.	PREVIOUS THERAPY (MO.)	DOSE (μG) OR (U.)	DOSE INTERVAL (WK.)	RESULTS BEFORE FOLIC ACID			DURATION (MO.) OF FOLIC ACID 5/0 MG. Q.D. AND—	RESULTS AFTER FOLIC ACID			DIFFERENCE BEFORE AND AFTER FOLIC ACID		
				R.B.C. (X 10^6)	HGB. (GM.)	M.C.V.		R.B.C. (X 10^6)	HGB. (GM.)	M.C.V.	R.B.C. (X 10^6)	HGB. (GM.)	M.C.V.
9	Normocytin (16)	60	3	4.38	13.1	100.5	Normocytin (7)	3.87	11.9	102.0	−0.51	−1.2	+1.5
16	Normocytin (16)	60	4	4.10	13.1	109.7	Normocytin (4)	4.58	13.6	97.2	+0.48	+0.5	−12.5
17	Normocytin (16)	60	3	4.15	12.9	101.2	Normocytin (6)	4.10	13.5	100.0	−0.05	+0.6	−1.2
18	Normocytin (16)	60	3	4.08	13.1	105.3	Normocytin (6)	3.62	11.9	114.6	−0.46	−1.2	−9.3
32	Normocytin (16)	60	3	4.38	13.8	100.9	Normocytin (7)	4.12	12.9	105.5	−0.26	−0.9	+4.6
34	Normocytin (11)	60	3	3.26	11.1	101.2	Normocytin (5)	3.44	11.0	101.8	+0.18	−0.1	+0.6
37	Normocytin (3)	60	1-3	4.76	14.7	102.9	Normocytin (7)	4.67	14.0	102.9	−0.09	−0.7	±0.0
23	Cryst. B_{12} (16)	22.5	3	3.91	12.4	106.1	Cryst. B_{12} (6)	4.25	12.9	100.0	+0.34	+0.5	−6.1
25	Cryst. B_{12} (16)	22.5	3	4.32	12.8	101.8	Cryst. B_{12} (7)	4.26	13.3	103.2	−0.06	+0.5	+1.4
29	Cryst. B_{12} (16)	22.5	3	4.23	13.9	105.2	Cryst. B_{12} (7)	4.21	13.8	108.4	−0.02	−0.1	+3.2
30	Cryst. B_{12} (7)	22.5	3	4.24	13.8	106.1	Cryst. B_{12} (7)	4.30	13.7	105.9	+0.06	−0.1	−0.2
42	Cryst. B_{12} (10)	22.5	3	4.25	14.4	112.9	Cryst. B_{12} (5)	4.83	14.9	102.5	+0.58	+0.5	−10.4
3	Liver (4)	30 u.	4	4.53	13.9	100.4	Liver (7)	4.46	13.6	99.6	−0.07	−0.3	−0.8
6	Liver (4)	30 u.	4	4.76	15.0	102.9	Liver (6)	4.49	14.9	104.9	−0.27	−0.1	+2.0
12	Liver (4)	30 u.	4	4.58	13.9	100.4	Liver (7)	4.31	13.5	105.4	−0.29	−0.4	+5.0
27	Liver (4)	30 u.	4	3.62	11.1	100.8	Liver (7)	3.66	10.7	95.8	+0.04	−0.4	−5.0
36	Liver (4)	30 u.	4	3.93	12.9	110.7	Liver (5)	3.95	12.3	106.3	+0.02	−0.6	−4.3
39	Liver (4)	30 u.	4	4.05	13.5	109.8	Liver (6)	4.67	13.8	100.5	+0.62	+0.3	−9.3

of the twenty-one patients, and improvement in erythrocyte levels occurred in every patient, sometimes dramatically. With the exception of the macrocytosis then, the administration of the vitamin B_{12} concentrate at regular intervals averaging 3 μg daily was adequate antianemia therapy. No patient in this or any other group had any evidence of development or progression of lingual or neurologic involvement during the course of observation.

Improvement also occurred after changing from treatment with the vitamin B_{12} concentrate to crystalline vitamin B_{12} in the same dosage averaging 1 μg daily given at intervals of three or four weeks. To rule out the possibility that a commercial excess over and above that stated on the label of the crystalline material was present, random samples of both the concentrate (Normocytin, Lederle) and the crystalline material (Betalin 12, Lilly) were reassayed by the respective manufacturers, and the content of vitamin B_{12} activity was found to be as labeled. In addition, the crystalline material was assayed in another laboratory, and the results agreed with those of the manufacturer and the label.[4] Standard microbiological assays, accepted as being reliable indicators of vitamin B_{12}, were employed.[5] These methods are very similar to and form the basis for the method adopted by the U.S. Pharmacopoeia as the official assay procedure.

The major difference between the two preparations, other than the fact that the concentrate was not a crystalline material, was the fact that the concentrate contains a mixture of vitamin B_{12} (cyanocobalamin) and vitamin B_{12b} (hydroxocobalamin). It has been demonstrated that vitamin B_{12b} has the same antipernicious anemia activity as vitamin B_{12} when tested in short-term experiments.[6, 7] As suggested previously,[3] it is possible that over a longer period of time, as in these studies, a lesser activity of vitamin B_{12b} might become more apparent than in shorter experiments on patients in relapse. At the present time, the majority of these forty-three patients being treated with vitamin B_{12} concentrate (Normocytin, Lederle) in a dose intended to average 1 μg daily given intramuscularly every three or four weeks in an effort to ascertain whether or not this is true. Unfortunately, it was learned subsequently that the product now contains a commercial excess so that material labeled to contain 30 μg per milliliter actually contains about 42 μg,[4] as a result of which the patients are receiving an average of about 1.3 μg daily rather than the 1 μg intended. Thirty-seven patients so treated for periods of three to thirteen months have shown no deterioration of erythrocyte values to date. If further observation reveals the concentrate to be effective in this dose, it can probably be concluded that the reason previous treatment with the smaller dose was inadequate was because the earlier preparations were less purified and the microbiologic assays less perfected, so that less vitamin B_{12} was contained than indicated by assay. In addition, recent comparison of the concentrate with crystalline material by the chick assay method has not demonstrated any significant difference when given by parenteral administration.[4] The crystalline material may have given a little better growth response at the higher levels tested (0.10 or 0.15 μg daily), but the concentrate gave equivalent values at the lowest level tested (0.05 μg daily) which should be the "most sensitive" region of the response curve. It

would appear at this time that any difference between the two preparations as indicated both by continued observation of these patients and by the chick assay are not significant.

These observations indicate that adequate doses of vitamin B_{12}, whether crystalline cyanocobalamin or concentrates containing both cyano- and hydroxocobalamin, will control pernicious anemia as effectively as purified liver extracts, and that addition of folic acid does not accomplish more than vitamin B_{12} or liver extract therapy by themselves. The purified liver extracts contain essentially no folic acid, or its derivative, folinic acid, but the possibility has existed that in addition to vitamin B_{12}, liver extracts contain some unknown substance(s) which might be of additional therapeutic value in pernicious anemia. The results of these observations do not support this concept. Whole liver and possibly some of the cruder liver extracts intended for oral use contain considerable amounts of folic acid which is not retained in the purified extracts intended for parenteral use. The "Wills' factor" is also present in some crude liver fractions,[8] but is not retained in the purified extracts as best shown by failure of the purified extracts to be affective in those macrocytic anemias which will respond to the cruder derivatives of whole liver.

The slightly elevated mean corpuscular volume (M.C.V.) values occurring in many of these patients was unexpected. It has been stated that macrocytosis is an indication of inadequate treatment even in the presence of normal erythrocyte counts,[9] and a top normal value for M.C.V. has been placed at about 95 cubic microns.[10, 11] Many of the slightly elevated values may be within the limits of error of the blood counting and hematocrit determinations, although if this were the case it might have been expected that an equal number of lower values would have been obtained. To test the significance of the macrocytosis, the erythrocyte counts of the treated patients were plotted against the hematocrit values (Fig. 1)* and compared with a series of "normal" patients without demonstrable hematopoietic disorder collected by another author.[11] It has been stated that in normal individuals changes in hematocrit determination bear a linear relationship to changes in erythrocyte count. In Fig. 1, x represents the hematocrit determination and y represents the erythrocyte count. The relation between these two values is represented mathematically:

$$x = a + by$$

where a and b are constants. The value a should presumably be zero, for without erythrocytes, the hematocrit would be zero. In plotting erythrocyte counts against hematocrit values, however, an extension of the line does not intercept the hematocrit ordinate at zero, a reflection of the well-known fact that the true hematocrit is not determined by the standard procedures.[10] Therefore, when the constants are derived by least squares, a is found to be greater than zero.

On the basis of widely accepted statements,[9-11] the relationship between erythrocyte counts and hematocrit readings in a group of adequately treated patients should be the same as in a group of "normal" individuals. In the two

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groups compared, the erythrocyte counts and hematocrit readings are linearly interrelated. For each series a straight line was fitted by the least squares method and these lines were compared (Fig. 1). The two lines are essentially parallel, indicating that the difference between the rate of increase in erythrocyte counts per increase in hematocrit is not statistically significant. However, the lines differ in that the erythrocyte counts of the treated patients are significantly lower than those of the "normal" individuals after adjusting for differ-

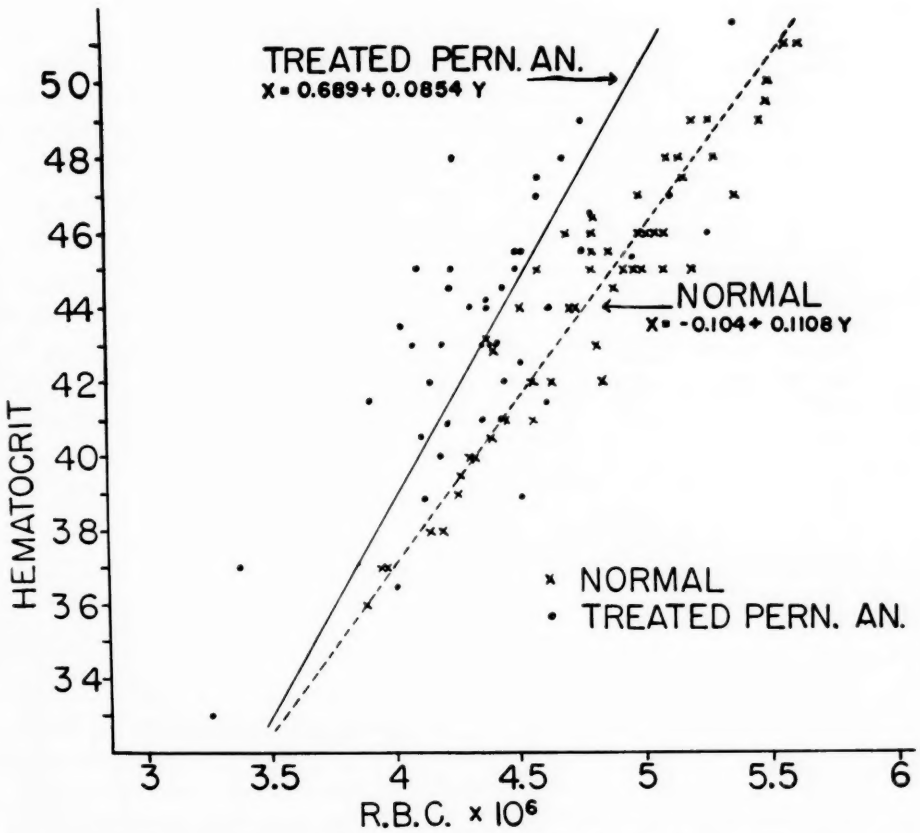

Fig. 1.—Hematocrit readings and red blood cell counts in 52 normal individuals (from the literature, reference 11) and 43 treated pernicious anemia patients. Regression lines are red blood cell counts on hematocrits.

ences in hematocrit readings. While differences in geographic location of the two groups, and sex and age differences could well account for different "normal" values for the erythrocytes and hematocrits, it has been shown that the two values bear a constant relationship to each other[9-11] so that this does not appear to be an adequate explanation of the differences observed in Fig. 1. Variation in laboratory procedure must be considered concerning which it can only be said that standard and similar procedures were used in the two series compared, although human error cannot be excluded entirely. The other pos-

sibility is that a true difference persists between the erythrocytes of treated patients and those of individuals without pernicious anemia.

This raises the question of whether the treatment given this group of patients was truly adequate. The fact that liver extract, given in the usual dosage, did not alter the macrocytosis, nor did the addition of folic acid to either vitamin B_{12} or liver extract treatment have any effect, indicates that vitamin B_{12} by itself was as effective as any other form of treatment used. Whether more frequent treatment with either vitamin B_{12} or liver extract would have eliminated the macrocytosis cannot be answered by these data. It has been customary to administer single injections of purified liver extract at intervals of four weeks, and in the authors' experience such treatment has maintained normal blood values and has prevented development or progression of neurologic and lingual lesions. We have been unable to find individual data in series of pernicious anemia patients treated for long periods of time which would permit calculation of the M.C.V., although such data presumably form the basis of the statements[9, 10] that macrocytosis does not persist when pernicious anemia is adequately treated.

If macrocytosis should be found subsequently to persist in a high proportion of treated cases, the possibility should be considered that some substance other than vitamin B_{12}, folic acid, or unknown materials in purified liver extracts is necessary for complete correction of the erythrocyte defect in pernicious anemia.

From the observations in this study, however, it appears that vitamin B_{12} in adequate dosage is no less effective than purified liver extract, and that the addition of folic acid does not augment the effect.

SUMMARY

Patients with pernicious anemia were given maintenance therapy with crystalline vitamin B_{12}, and a "concentrate" containing vitamin B_{12} and B_{12b} for periods up to sixteen months. In the dosages used, these substances were as effective as purified liver extract.

An average daily dose of 1 μg given intramuscularly at intervals of three or four weeks appeared to be sufficient to maintain normal erythrocyte levels and to prevent the development or progression of neurologic or lingual lesions.

In previous experiments, the concentrate was less effective than the crystalline preparation, but with the concentrates available now there is probably no significant difference.

Mild macrocytosis persisted in many of the patients and was not altered by the intramuscular administration of purified liver extract nor by the addition of daily oral doses of folic acid. Comparison of the treated patients with a series of "normals" from the literature indicates that the relationship of the erythrocyte level to hematocrit value in the two series is different, even when treated patients have high "normal" blood values.

This suggests the possibility that neither vitamin B_{12}, purified liver extract, nor folic acid given singly or in combination completely corrects the erythrocyte abnormality in pernicious anemia and suggests that some other substance(s) may be necessary in addition.

From the practical standpoint, however, treatment with sufficient amounts of vitamin B_{12}, B_{12} concentrates, or purified liver extract maintains normal erythrocyte levels and prevents development or progression of neurologic and lingual lesions.

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THE COMBINED ANTICONVULSANT ACTIVITY AND TOXICITY OF DILANTIN AND N-METHYL-5-PHENYLSUCCINIMIDE

GRAHAM CHEN, SC.D., M.D., AND CHARLES R. ENSOR, M.S.
DETROIT, MICH.

NO THERAPEUTIC agent is yet available which is efficacious and nontoxic for the treatment of both grand mal and petit mal epilepsy. In the management of these mixed seizures in the same patient, therefore, the therapy resorted to is a combination of two drugs, one for each disorder. N-methyl-5-phenylsuccinimide (PM334) has recently been shown to be a very effective drug for petit mal epilepsy;[1] it is to be expected that PM334 will be employed jointly with Dilantin, which is more specific against grand mal, in the control of mixed types of seizures. As in any therapy with a combination of drugs, it is important to know whether or not these agents, when given together, will be altered in their therapeutic effectiveness and toxicity. In searching for such information, the present investigation on the combined anticonvulsant activity and toxicity of Dilantin and PM334 was conducted. It is contemplated that the results obtained with the two agents from electrically and chemically induced convulsions will be coordinated with the clinical findings from their combined therapy in the management of mixed grand and petit mal seizures. Such a study may reveal also the reliability of the present laboratory methods in the evaluation of potential antiepileptic drugs.

EXPERIMENTAL

The combined anticonvulsant effect of Dilantin and PM334 was investigated both on electrically and pentylenetetrazol (Metrazol) induced convulsions by oral administration. The antielectroshock activity was determined in cats by the method of Putnam and Merritt[2] and in mice by the supramaximal electroshock method of Toman, Swinyard, and Goodman[3] with procedures as previously given.[4] A minimal anticonvulsant dose of each compound was used to obtain their combined effect in the cat; various ratios of the two in proportion to their individual PD_{50}s were employed to determine their joint anticonvulsant activity in the mouse.[5]

The determination of their anti-Metrazol effect was conducted in three groups of ten rats each, which were challenged with three doses of the convulsant. The quantities of Metrazol were so chosen as to produce convulsions in 20 to 80 per cent of the animals in each group.

The acute lethal toxicities of Dilantin and PM334 were determined in mice, using twenty or forty animals per dose, in a period of five days. A difference of 20 per cent, or a ratio of 1.2 between each dose level was utilized to obtain a graded lethal toxicity in six groups of mice. The doses of Dilantin and PM334 in the combination were made in proportion to their individual LD_{50}s.

The 50 per cent protective dose, convulsive dose, lethal dose, and their standard errors were computed graphically.[6]

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TABLE I. THE EFFECT OF DILANTIN AND PM334 ON ELECTRICALLY INDUCED CONVULSIONS IN CATS BY THE METHOD OF PUTNAM AND MERRITT

DAY OF INGESTION	DILANTIN			PM334			DILANTIN + PM334		
	CAT NO.	MG./KG.* ORAL	ACTIVITY	CAT NO.	MG./KG.* ORAL	ACTIVITY	CAT NO.	MG./KG.* ORAL	ACTIVITY
1st	A	8	<1+	D	100	1+	G	8 + 100	1+
2nd		8	1+		100	1+		8 + 100	2+
3rd		8	2+		100	1+		8 + 100	3+
1st	B	8	<1+	E	100	1+	H	8 + 100	1+
2nd		8	1+		100	1+		8 + 100	2+
3rd		8	2+		100	1+		8 + 100	3+
1st	C	8	1+	F	100	0	I	8 + 100	0
2nd		8	1+		100	<1+		8 + 100	1+
3rd		8	1+		100	<1+		8 + 100	4+

*Nontoxic doses.

RESULTS AND COMMENT

The data in Table I indicate that the anticonvulsant potency of Dilantin is approximately twelve times that of PM334 as determined on a weight basis by their ability to raise the electric shock convulsion threshold in cats. The effect of Dilantin was slow and cumulative, while that of PM334 was not. However, the combination of the two gave merely an additive effect; the increase in anticonvulsant activity following daily administration was apparently due to the action of Dilantin.

Results of similar nature were obtained in mice by the abolition of the extensor tonic component of the convulsion to a supramaximal electroshock stimulus (24 ma.). With this test, PM334 is only one twentieth as effective as Dilantin (Table II). The straight line in Fig. 1, which was made by plotting the PD_{50}s of the various combinations of Dilantin and PM334 against their individual doses,[5] shows an additive effect of the combination.

Judging from the relative antielectroshock activities of the two agents obtained in the cat and the mouse by the two tests, and assuming the therapeutic dose of Dilantin to be 0.1 Gm. three times daily, the clinical dose of

TABLE II. THE ANTICONVULSANT EFFECT OF DILANTIN AND PM334 ON ELECTROSHOCK CONVULSIONS IN MICE BY THE METHOD OF TOMAN, SWINYARD, AND GOODMAN

DILANTIN (A)		PM334 (B)		(1) 0.3 PD_{50}A+ 0.7 PD_{50}B		(2) 0.5 PD_{50}A+ 0.5 PD_{50}B		(3) 0.7 PD_{50}A+ 0.3 PD_{50}B	
MG./KG.	NO. PROT. / NO. INJ.	MG./KG.	NO. PROT. / NO. INJ.	MG./KG.	NO. PROT. / NO. INJ.	MG./KG.	NO. PROT. / NO. INJ.	MG./KG.	NO. PROT. / NO. INJ.
8.3	1/10	177	1/10	2.6+ 134.0	0/10	4.4+ 95.6	0/10	6.2+ 57.4	1/10
10.0	1/10	212	2/10	3.2+ 161.0	2/10	5.3+ 115.0	1/10	7.4+ 69.0	2/10
12.0	3/10	254	1/10	3.8+ 193.2	5/10	6.4+ 138.0	4/10	8.9+ 82.8	4/10
14.4	7/10	305	7/10	4.6+ 232.0	7/10	7.7+ 165.8	7/10	10.7+ 99.2	7/10
17.3	9/10	366	8/10	5.5+ 279.0	9/10	9.2 198.2	10/10	12.8+ 119.0	8/10
PD_{50} ± S.E.									
12.8 ± 0.5		277 ± 11		4.0 ± 0.2A 203.0 ± 8.6B		6.7 ± 0.2A 145.0 ± 5.2B		9.5 ± 0.4A 88.0 ± 3.8B	

PM334 for grand mal epilepsy would be from 3.6 to 6.0 grams. These quantities of PM 334 approximate the maximum tolerated doses in man.[1] On the other hand, it will be of practical interest to test the therapeutic effect of PM334 in epilepsy supplementary to Dilantin therapy. We have observed in a girl in our care, who has a history of injury at birth, that, while Dilantin (a single dose of 135 mg. in the evening) has controlled her major seizures, the

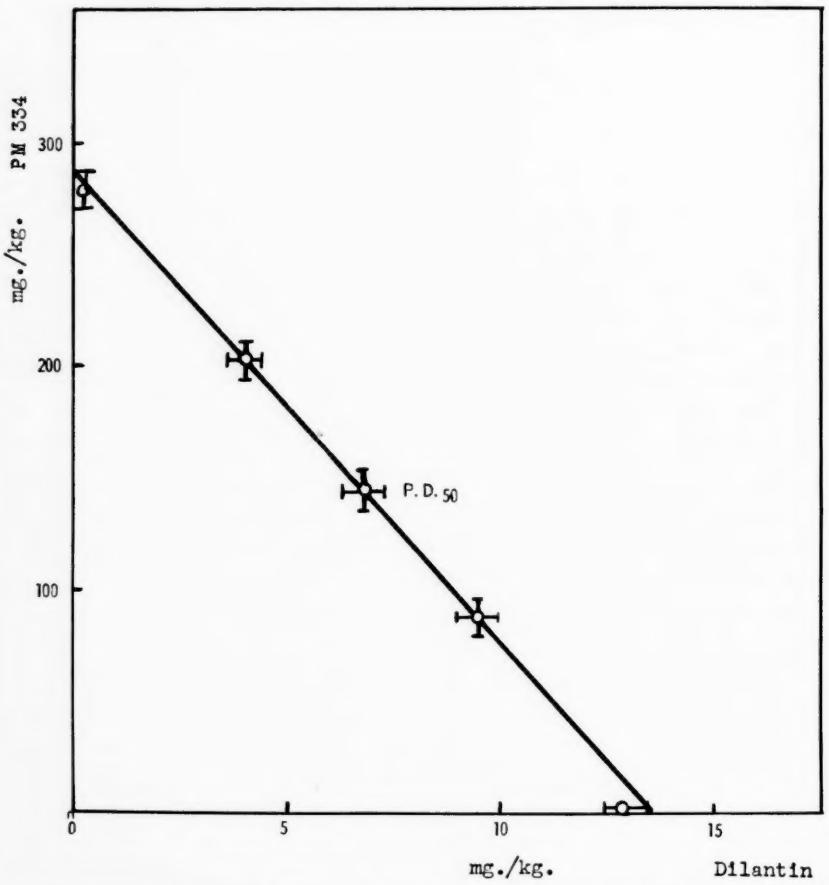

Fig. 1.—Fifty per cent protective doses of various combinations of Dilantin and PM334 against electroshock convulsions in mice. The straight line indicates an additive effect of the two compounds. The circles are experimentally determined values; their standard errors are marked by the horizontal lines.

addition of PM334 (600 mg. after breakfast) has markedly improved her mental attitude, alertness, and efficiency in speech. A combined therapy of Dilantin and PM334 may be indicated also in seizure states with behavior disturbances.

Whereas the two agents additively suppressed electrically induced seizures, Dilantin was ineffective both alone and in combination with PM334 in protecting rats from convulsions by Metrazol. In fact, a large dose of Dilantin

(200 mg./kg.) reduced the anti-Metrazol effect of PM334 (Experiment 1, Table III). This is evidently due to the stimulating action of Dilantin at high dose levels.[7] In view of the report that Dilantin gave some anti-Metrazol effect upon chronic administration,[8] the combined anti-Metrazol activity of PM334

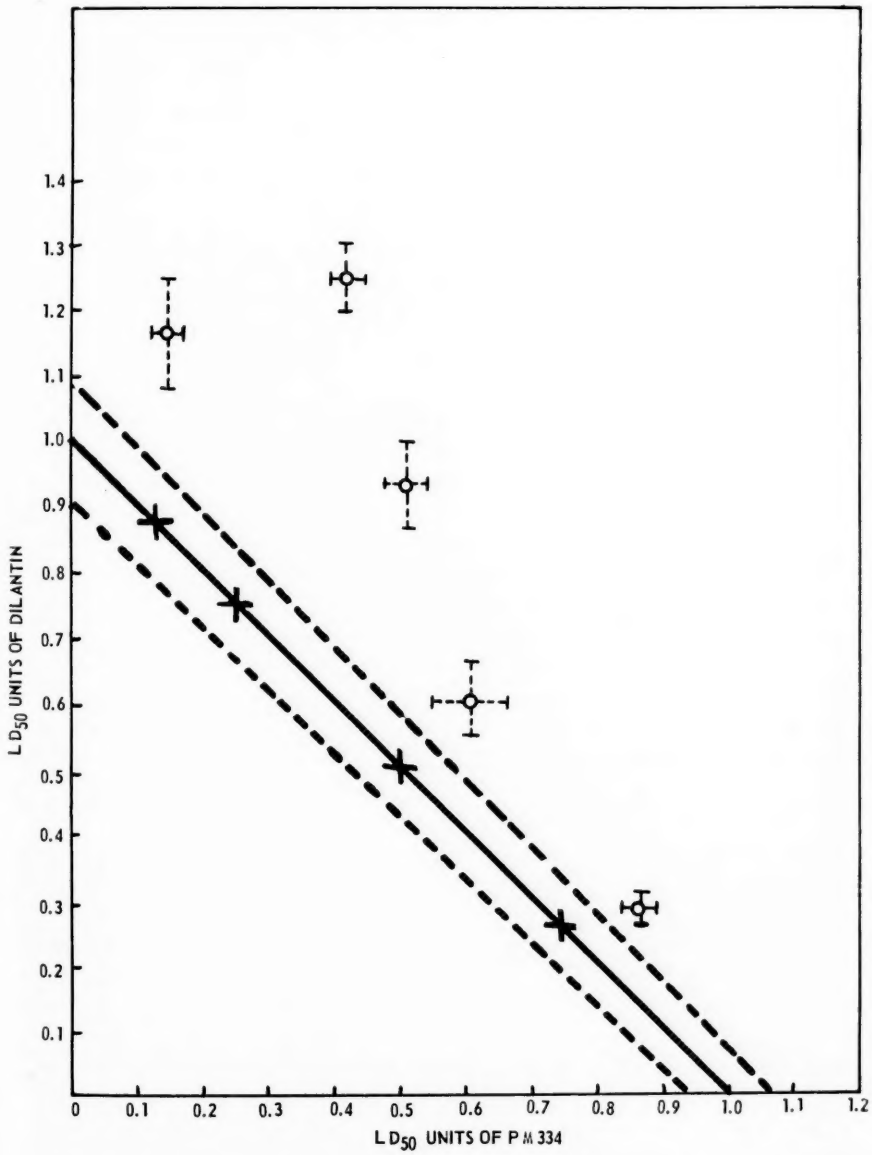

Fig. 2.—The combined lethal toxicity of Dilantin and PM334 in mice by oral administration. The crosses represent various mixtures of Dilantin and PM334 made up in proportion to their LD_{50}s; the solid line drawn through these points indicates an additive joint effect. The dotted lines represent the margin of scattering of ±2 standard errors from the solid line as calculated from LD_{50} determinations of Dilantin and PM334. The circles are experimentally determined values; their standard errors (±2) are represented by the horizontal lines.

TABLE III. THE ANTI-METRAZOL EFFECT OF DILANTIN AND PM334 IN RATS

EXPERIMENT 1			EXPERIMENT 2		
DILANTIN MG./KG.	PM334 (ORAL)*	METRAZOL MG./KG. (IM) CD_{50} ± S.E.	DILANTIN MG./KG. (TWICE DAILY)	PM334 (ORAL)†	METRAZOL MG./KG. (IM) CD_{50} ± S.E.
0	0	50.7 ± 1.2	0	0	49.0 ± 1.2
25	0	50.1 ± 1.3	50	0	54.7 ± 1.7
200	0	49.0 ± 1.2	0	60	62.4 ± 4.2
0	100	82.2 ± 2.5‡	50	60	63.1 ± 5.0
25	100	80.4 ± 2.4	0	90	79.4 ± 2.0
50	100	80.4 ± 2.4	50	90	78.5 ± 2.4
200	100	58.9 ± 2.1‡	0	120	92.5 ± 2.8
			50	120	85.9 ± 2.2

*One-half hour before Metrazol.

†Dilantin given twice daily for four days; Metrazol injected sixteen hours after the last ingestion of Dilantin and one-half hour after PM334.

‡The difference is significant, P = 0.01.

TABLE IV. ACUTE LETHAL TOXICITY OF DILANTIN AND PM334 IN MICE BY ORAL ADMINISTRATION

Experiment A

DILANTIN		PM334		DILANTIN + PM334* 75% 25%		DILANTIN + PM334* 50% 50%		DILANTIN + PM334* 25% 75%	
MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.
208	11/20	868	8/20	182 + 245	4/20	100 + 408	1/20	50 + 613	5/40
250	13/20	1042	15/20	218 + 294	2/20	120 + 490	7/20	61 + 735	8/40
300	16/20	1250	17/20	261 + 353	8/20	144 + 588	8/20	73 + 882	28/40
360	17/20	1500	19/20	314 + 423	21/40	173 + 706	13/20	87 + 1058	19/20
432	17/20	1800	20/20	376 + 508	13/20	208 + 847	18/20		
				452 + 610	17/20				
LD_{50} ± 2 S.E.									
Dilantin, 242 ± 22		PM334, 980 ± 66		Dilantin + (304 ± 19) + PM334 (410 ± 26)		(146 ± 12) + (600 ± 54)		(70 ± 2) + (851 ± 24)	

Experiment B

DILANTIN		PM334		DILANTIN + PM334* 87.5% 12.5%		DILANTIN + PM334* 62.5% 37.5%	
MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.	MG./KG.	NO. DEAD / NO. INJ.
202	14/20	817	6/20	212 + 123	6/20	151 + 368	5/20
242	11/20	980	10/20	254 + 147	16/20	182 + 441	6/20
290	16/20	1176	13/20	305 + 176	12/20	218 + 529	13/20
				366 + 212	18/20	261 + 635	18/20
				440 + 254	20/20	314 + 762	18/20
LD_{50} ± 2 S.E.							
Dilantin, 216 ± 17		PM334, 960 ± 60		Dilantin, 252 ± 18 PM334, 145 ± 4		Dilantin, 202 ± 14 PM334, 492 ± 34	

*The mixtures of Dilantin and PM334 were made up in percentages of their LD_{50}s and the proportions thereof.

and Dilantin following daily administration of the latter (four days) was investigated. The results are given in Table III (Experiment 2). The dose of Dilantin was approximately twice its antielectroshock PD_{50} in rats.[9] Dilantin did not produce an anti-Metrazol effect by this ingestion procedure either alone or in the combination. The activity of the combination is accounted for by the action of PM334. From the results of these experiments, it may be concluded that Dilantin and PM334 when administered jointly exert their central nervous action separately.

The acute lethal toxicities of Dilantin, PM334, and their combinations in mice are shown by the data in Table IV. The $LD_{50} \pm$ S.E. of the combinations are significantly greater than the calculated doses for an additive joint effect; this is shown graphically in Fig. 2 by the values above the lines for a similar additive action. It appears that the action of one is mutually antagonized by that of the other in their acute toxicity. The fact that Dilantin is primarily a central nervous system stimulant at large doses, and that PM334 is a depressant at all dose levels, may account for the greater antagonism between the two at doses above half of the LD_{50} of Dilantin (as shown by the points on the left side of the graph).

SUMMARY

The anticonvulsant effect and acute lethal toxicity of Dilantin and N-methyl-5-phenylsuccinimide (PM334), administered jointly, were investigated. The combined antielectroshock activity was found to be additive, while their combined acute toxicity was antagonistic in nature. Dilantin is ineffective, either alone or in combination with PM334, against Metrazol-induced convulsions; however, its presence in nontoxic doses does not interfere with the anti-Metrazol effect of PM334. The conclusion is that the two act separately on the central nervous system. Comments are given regarding a combined therapy of Dilantin and PM334 in the management of mixed grand mal and petit mal epilepsy and of seizure states with behavior disturbances.

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POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES STORED IN A SOLUTION OF ETHYLENEDIAMINE TETRAACETIC ACID AND DEXTROSE

CHARLES C. SPRAGUE, M.D.,* JOHN B. SHAPLEIGH, M.D.,** SUE MAYES, B.S., ROBERT D. LANGE, M.D., AND CARL V. MOORE, M.D.
ST. LOUIS, MO.

IN BROAD transfusion programs designed to meet defense as well as peacetime requirements, there is great need for a single preservative solution which would: (a) prolong the period during which blood may be stored, and (b) permit the collected blood to be used either for transfusion or for conversion into dried plasma without change in the current lyophilizing techniques. The late Dr. Russell L. Haden suggested that the di-sodium salt of ethylenediamine tetraacetic acid (Sequestrene NA2,† di-sodium EDTA) should be evaluated to see whether its substitution for citrate might provide a partial solution to this problem. This suggestion was prompted by several observations. Dyckerhoff and his associates[1] found Sequestrene to be ten times more effective than citrate as an anticoagulant. Proescher[2] reported that it was less damaging to the cellular elements of the blood in vitro and seemed to preserve them better than did sodium citrate or the oxalates. Survival of transfused red cells in recipients, however, was not described. Platelets appear to be well preserved by EDTA: Dillard and his associates[3] have used a solution of 1 Gm. di-sodium EDTA and 0.7 Gm. sodium chloride in 100 ml. of distilled water for collecting blood to be used for platelet transfusions, nine parts of blood being added to one part of the Sequestrene solution. The authors have employed the same solution as well as 4.5 per cent di-sodium EDTA (isotonic) for collection of blood to be used for platelet transfusions. No evidence of toxicity was noted in any of these transfusions or when twenty transfusions[2] were given of whole blood collected in a solution containing di-sodium EDTA and dextrose (1.5 Gm. of di-sodium EDTA dissolved in 100 ml. of 5 per cent dextrose for each 500 ml. of blood). As a background study for further work with di-sodium EDTA, therefore, it was necessary to determine that post-transfusion survival of erythrocytes stored in a Sequestrene-dextrose solution for one to twenty-eight days was at least as good as with acid citrate dextrose (ACD) solution. The present report provides that evidence and indicates that additional observations with longer periods of storage should be made.

From the Department of Medicine, Washington University School of Medicine.

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*Commonwealth Fellow.

**Trainee of the National Cancer Institute.

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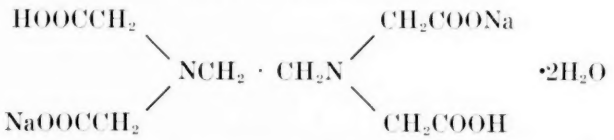
Ethylenediamine tetraacetic acid was synthesized by Bersworth in 1927 and has the following structural formula:

It is a white substance, almost insoluble in water or the common organic solvents; however, the di-, tri-, and tetra-sodium salts are readily soluble in water.[4] The di-sodium salt (Sequestrene NA2) has the following formula:



These salts do not hydrolyze in aqueous solution and are stable over a wide range of temperature and pH. They deionize alkali earths and heavy metals, and act, therefore, as water-soluble ion exchangers. The pH of an aqueous solution is approximately 5. We found a 4.5 per cent solution to be isotonic for red cells.

Sequestrene is poorly absorbed from the gastrointestinal tract, approximately 96 per cent of an oral dose being excreted unchanged in the feces.[2] Popovici and co-workers[5] found it possible to inject intravenously 2,000 mg. per

TABLE I. SURVIVAL OF TRANSFUSED ERYTHROCYTES DURING FIRST 72 HOURS AFTER TRANSFUSION—COMPARISON OF OBSERVED WITH EXPECTED NON-AGGLUTINABLE COUNT

SUBJECT	SOLUTION	PERIOD OF STORAGE (DAYS)	INITIAL OBSERVED NONAGGLUTINABLE COUNT† (PER MM.^{3})	% OF EXPECTED COUNT CALCULATED ON BASIS OF BLOOD VOLUME DETERMINED BY: T-1824 DYE METHOD UNCORRECTED (%)	T-1824 DYE METHOD CORRECTED (HEMATOCRIT × 0.95 × .87)* (%)	PLASMA VOL. FROM T-1824 DYE METHOD, RED CELL VOL. FROM Fe^{59}-TAGGED CELL METHOD (%)
1. F	Seq.-Dextrose‡	1	825,000	113	99	101
2. C	Seq.-Dextrose	1	1,043,000	134	118	119
3. F	ACD	1	747,000	128	114	113
4. K	ACD	1	878,000	132	109	114
5. H	Seq.-Dextrose	10	642,000	100	89	87
6. H	Seq.-Dextrose	10	760,000	101	89	90
7. S	ACD	10	653,000	114	102	99
8. S	ACD	10	684,000	113	105	101
9. G	Seq.-Dextrose	21	814,000	114	98	107
10. P	Seq.-Dextrose	21	703,000	109	96	108
11. S	ACD	21	695,000	142	120	118
12. Mc.	ACD	21	568,000	101	91	82
13. P	Seq.-Dextrose	28	498,000	90	77	Not Determined
14. E	Seq.-Dextrose	28	679,000	93	83	82
15. S	ACD	28	455,000	75	65	63
16. T	ACD	28	652,000	85	74	Not Determined

*Factor of 0.95 to correct for plasma trapped with red cells in determination of hematocrit; factor of 0.87 to convert venous hematocrit to hematocrit of total body fluid.[8]

†Average of counts taken twenty-four, forty-eight, and seventy-two hours after transfusion.

‡Sequestrene NA2-Dextrose.

kilogram into rabbits over a period of three hours before fatal hypocalcemic tetany occurred. It seems unlikely, therefore, that toxic levels would be attained with multiple transfusions.

MATERIAL AND METHODS

Five hundred milliliters of blood were collected by gravity fom 32 healthy Group O donors and stored at 4° to 6° C. for one, ten, twenty-one, and twenty-eight days. Sixteen of the collections were made into bottles each of which contained 1.5 Gm. Sequestrene NA2

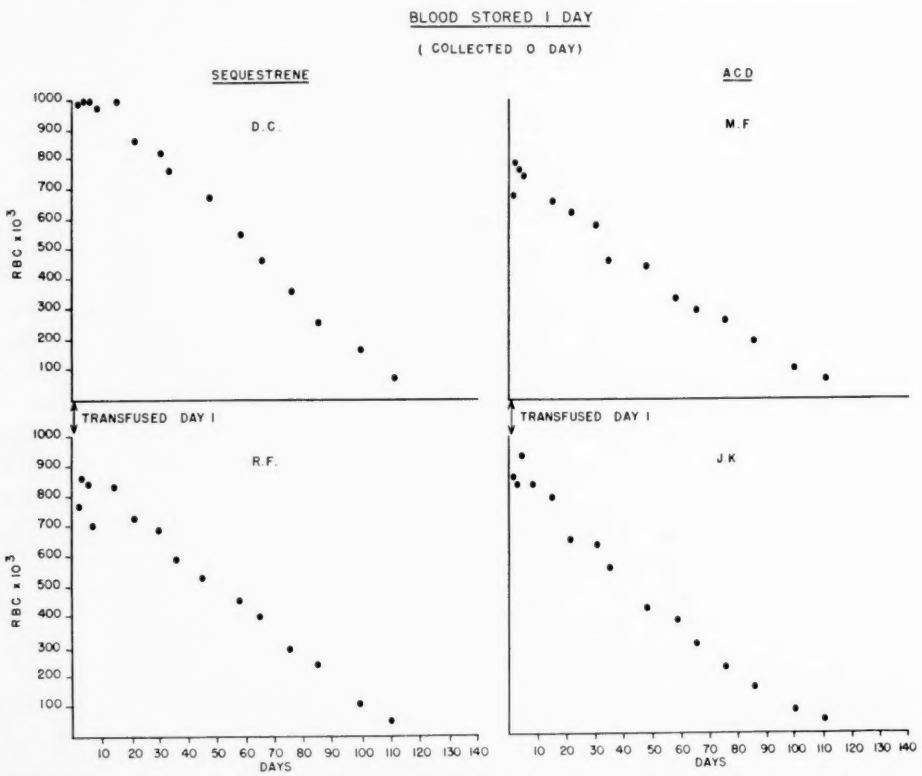


Fig. 1.—Comparison of the survival of erythrocytes stored for one day at 4 to 6° C. in (*a*) Sequestrene NA2-Dextrose solution, and (*b*) ACD solution. Group O healthy donors and Group A healthy recipients.

dissolved in 100 ml. of 5 per cent glucose. The remaining 16 units were collected into bottles containing an acid citrate dextrose solution of the following formula:

1.6 Gm. trisodium citrate
0.56 Gm. citric acid
1.5 Gm. dextrose
Water to 75 ml.

The 16 recipients were healthy medical students, all of whom belonged to Group A. Two units of blood were transfused into each recipient. For each of the four storage periods, therefore, there were two recipients who received blood preserved in the Sequestrene NA2 dextrose solution, and two who received blood preserved in the acid citrate dextrose solution.

Survival of the transfused erythrocytes was determined by the differential agglutination technique of Ashby, as modified by Dacie and Mollison[6]. The same batch of high titer

anti-A serum was used throughout the study. The serum was packaged in capillary tubes and kept frozen until used. Blank values for the recipients varied from 4,000 to 22,000 per cubic millimeter. Duplicate counts were made by the same three observers at the time of each determination, and the results were averaged.

In order to estimate the initial survival of the transfused cells, the following data were obtained. The blood and transfusion equipment were weighed before and after transfusion to obtain the weight of blood transfused, and the specific gravity of a mixed sample of the blood was determined. From these two figures, the volume of blood transfused was calculated.

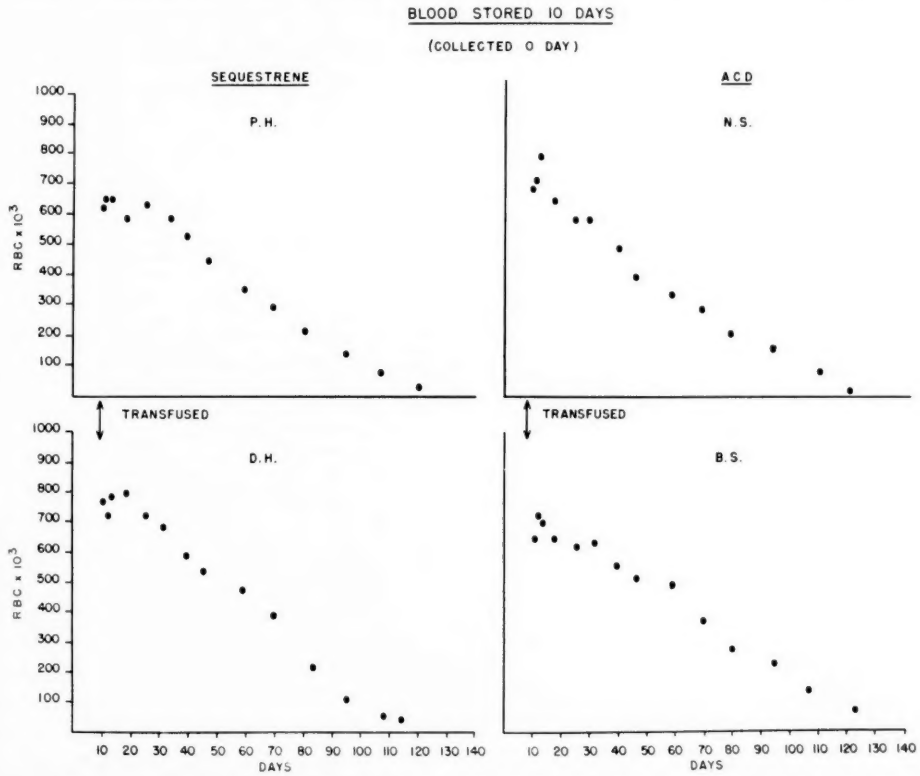


Fig. 2.—Comparison of the survival of erythrocytes stored for ten days at 4 to 6° C. in (*a*) Sequestrene NA2-Dextrose solution, and (*b*) ACD solution. Group O healthy donors and Group A healthy recipients.

Duplicate red cell counts made with equipment standardized by the U.S. Bureau of Standards were also performed on the mixed samples of blood; it was then possible to calculate the total number of erythrocytes transfused.

Blood volume determinations on the recipients were made by the Evans Blue dye (T-1824) method and by injecting compatible red cells tagged with Fe^{59}. With the dye method, the patient was placed in the recumbent position. After a brief rest period, a sample of venous blood was collected without stasis from the anticubital vein, and then 5 ml. of a 0.5 per cent aqueous solution of the dye* was injected intravenously; samples of venous blood were drawn without stasis through 18-gauge needles ten and twenty minutes following the injection of dye. The three samples were placed in graduated centrifuge tubes, capped with parafilm, and spun at 3,000 r.p.m. for thirty minutes to determine the hematocrit. The supernatant plasma was aspirated and the dye concentrations in plasma were read with a Beck-

*Packaged in 5 ml. ampules by William R. Warner & Co., Inc., Division of Warner-Hudnut, Inc., New York, N. Y.

man spectrophotometer at a wave length of 625 Angstrom units. These concentrations were plotted and extrapolated to zero time.

The Fe^{59}-tagged cells were obtained from a patient with polycythemia vera who had received repeated doses of Fe^{59} over a period of weeks. Fifty milliliters of freshly drawn blood from this patient was injected into each recipient immediately prior to the dye injection, and the activity in aliquots of the ten- and twenty-minute specimens mentioned above was determined by the method used in this laboratory.[7] From the blood volume of the recipient and the value for the total number of red cells given in the transfusion, it was

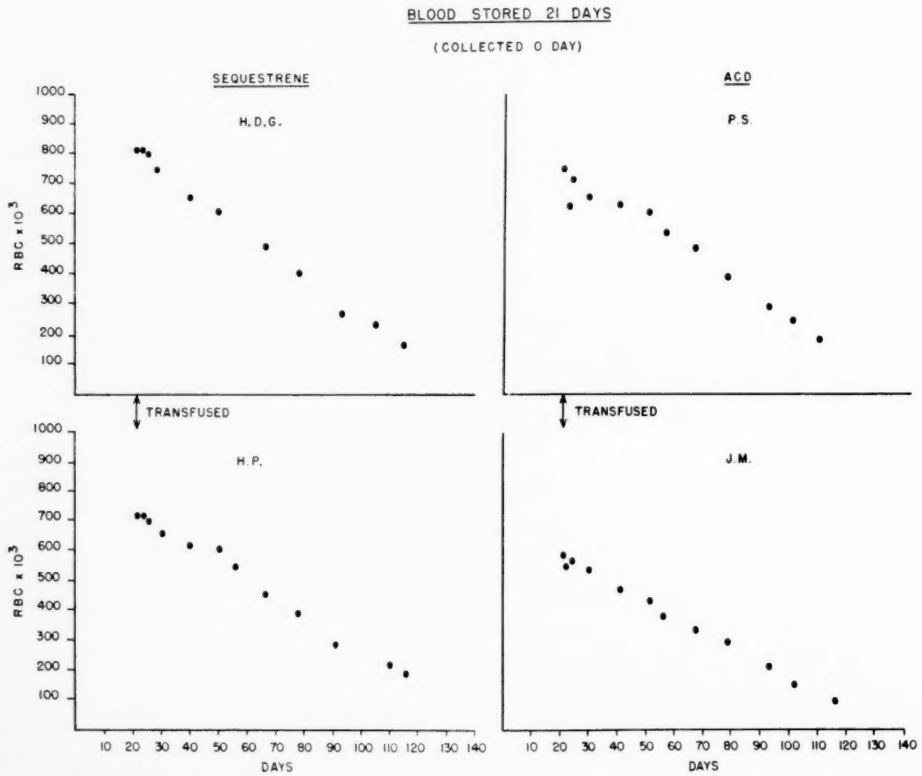


Fig. 3.—Comparison of the survival of erythrocytes stored for twenty-one days at 4 to 6° C. in (*a*) Sequestrene NA2-Dextrose solution, and (*b*) ACD solution. Group O healthy donors and Group A healthy recipients.

possible to calculate the number of transfused red cells which should have been present per cubic millimeter of recipients' blood if all of the red cells had survived. This figure was then compared with the observed value obtained during the initial post-transfusion period. No significant decrease occurred during the first three days. Therefore, rather than rely on a single count obtained twenty-four hours following the transfusion, counts were performed after twenty-four, forty-eight, and seventy-two hours, and the average of these three was used as the initial survival figure.

RESULTS

The rate at which transfused cells disappeared from the circulation of the recipients is presented graphically in Figs. 1 to 4. In the graphs, day "O" represents the day on which the transfused blood was drawn. There was no

significant difference in the slope of the curves for the red cells preserved in the two different solutions. Some of the transfused erythrocytes survived for approximately one hundred twenty days in every instance. The cells still present at the end of seventy-two hours therefore survived normally even after storage periods of as long as twenty-eight days.

Of equally great importance, however, was the percentage of transfused cells remaining in the circulation during the first seventy-two hours. This

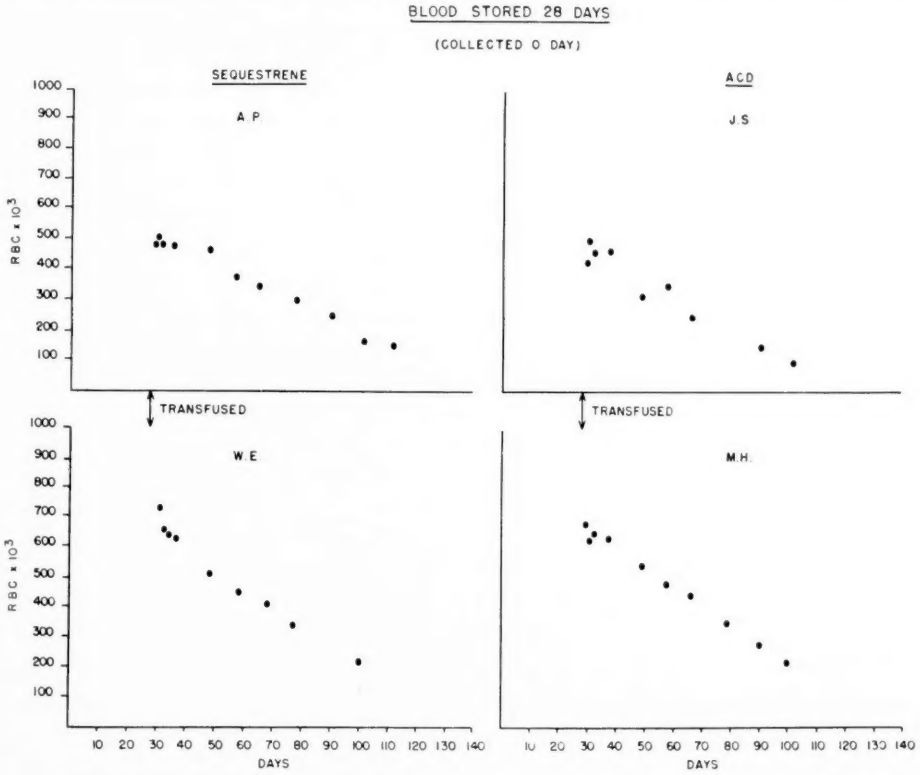


Fig. 4.—Comparison of the survival of erythrocytes stored for twenty-eight days at 4 to 6° C. in (*a*) Sequestrene NA2-Dextrose solution, and (*b*) ACD solution. Group O healthy donors and Group A healthy recipients.

figure varied with the method used for determining the blood volume, and is calculated in Table I in three different ways: (a) from the uncorrected blood volume as obtained by the dye method; (b) from the T-1824 blood volume corrected[8] for plasma trapped in the centrifuged red cells producing an artificially high hematocrit (0.95) and for conversion of venous hematocrit to body hematocrit (0.87); and (c) from blood volume obtained by adding the red cell mass as measured by the Fe^{59}-tagged erythrocytes to the plasma volume as obtained from the dye method. With each type of calculation, the actual observed count during the first seventy-two hours following transfusion was greater in some instances than the theoretically expected count if 100 per cent of the transfused cells had survived. The reason for these high values is not evident, and the

figures should be used merely as a standard of reference for comparison between the two preservative solutions and for the four different storage periods. The data indicate clearly that: (1) there was no significant difference in the initial seventy-two hour survival of blood collected and stored in ACD or Sequestrene NA2-dextrose solution; (2) the survival was excellent with storage periods of one, ten, and twenty-one days; survival after twenty-eight days of storage was still good, although it had definitely decreased.

One additional observation should be described. Blood collected in the Sequestrene NA2-dextrose solution and stored for thirty-five days has been transfused to one recipient. The observed unagglutinable cell count during the initial seventy-two hour post-transfusion period was approximately 65 per cent of the theoretical count, and the cells surviving that period in the circulation had an apparently normal life span. No transfusion of ACD blood stored for 35 days has been made so that comparison is not possible.

COMMENTS AND SUMMARY

Blood collected in a solution containing 1.5 Gm. of Sequestrene NA2 dissolved in 100 ml. of 5 per cent glucose and stored for periods up to twenty-eight days at 4 to 6° C. survived in vivo after transfusion as well as did blood collected in ACD solution. The amounts of Sequestrene NA2 and glucose used were the same as had been employed by Proescher, and are probably excessive. Further study should be made to determine optimum concentrations, and to discover whether preservation is good enough with Sequestrene NA2 to permit transfusion of blood after thirty-five days of storage.

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VARIATION IN COMPLEMENT FIXATION ANTIGEN PRODUCTION BY DIFFERENT STRAINS OF HISTOPLASMA CAPSULATUM GROWN ON TWO MEDIA

JOSEPH SCHUBERT, PH.D., LIBERO AJELLO, PH.D., SARAH STANFORD, A.B., AND VIRGINIA Q. GRANT, A.B.
ATLANTA, GA.

HISTOPLASMIN is the crude antigenic filtrate of liquid culture media in which *Histoplasma capsulatum* has been grown for varying periods of time. It has proved useful in skin testing surveys, and has been utilized by several workers either routinely or experimentally as an antigen in complement fixation tests for histoplasmosis (Van Pernis, Benson, and Hollinger,[1] Salvin,[2] Tennenberg and Howell,[3] and Furcolow, Bunnell, and Tennenberg.[4] In addition, for the past three years the C. D. C. Laboratories have been using histoplasmin as a complement fixation antigen. These investigators and others have employed either the filtrate from a single strain of *H. capsulatum* or the pooled filtrates of a number of strains, although no experimental evidence favoring the use of one type over the other exists. The present study was initiated to evaluate the relative merits of multiple and single-strain lots of histoplasmin in the complement fixation test.

In addition, prompted by the findings of Salvin and Hottle,[5] the ability of a glycogen medium to produce rapidly potent lots of histoplasmin was investigated.

MATERIALS AND METHODS

Antigen Preparation.—Ten stains of *H. capsulatum*, all human isolates, from the culture collection of the Mycology Laboratory (Nos. 28, 99, 105, 208, A 228, 232, A 233, A 235, 267, 430) were grown for six months on Smith's, et al.[6] modified Bureau of Animal Industry Tuberculin Medium (henceforth designated as the Smith coccidioidin or SC medium) and for 9 weeks on Salvin and Hottle's[5] glycogen medium[2] (hereinafter designated as the SG Medium). The incubation periods employed with the respective media approximated those used by Howell[7] and Salvin and Hottle.[5]

1. Formula: Smith's coccidioidin medium—SC

l-Asparagine	7.0 Gm.
Ammonium chloride C.P. (NH_4Cl)	7.0 Gm.
Glucose (Cerelose)	10.0 Gm.
Glycerin C.P.	25.0 Gm.
Magnesium sulfate C.P. ($MgSO_4 \cdot 7H_2O$)	1.5 Gm.
Dipotassium phosphate C.P. $K_2H\ PO_4$	1.31 Gm.
Sodium Citrate C.P. $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}\ H_2O$	0.9 Gm.
Ferric citrate (Scales) U.S.P.	0.3 Gm.
Distilled water to make	1,000.0 ml.

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2. Formula: Salvin and Hottle's glycogen medium—SG

l-Asparagine	14.0 Gm.
Glycogen	10.0 Gm.
Glycerin C.P.	25.0 Gm.
Magnesium sulfate C.P. ($MgSO_4 \cdot 7H_2O$)	1.5 Gm.
Dipotassium phosphate C.P. $K_2H\ PO_4$	1.31 Gm.
Sodium citrate C.P. $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}\ H_2O$	0.9 Gm.
Ferric Citrate (Scales) U.S.P.	0.3 Gm.
Distilled water to make	1,000.0 ml.

After inoculation with a small amount of mycelium from a 2-week-old subculture of each strain, the 4-liter flasks containing 1 liter of nutrient were incubated at room temperature (20 to 25° C.) in a dark closet. At the end of their respective incubation periods the broths were decanted, Seitz-filtered, tested for sterility, and stored at 4 to 5° C. following the addition of merthiolate (1:10,000). Due to contamination, the flasks of the SC medium inoculated with strains 208 and A 233 and the SG medium with strain 105 were not available for use in this experiment.

Antiserum Preparation.—Antisera for each of the ten strains of *H. capsulatum* were prepared according to the method described by Saslaw and Campbell.[8] All ten strains of *H. capsulatum* were converted to the yeast phase by repeated subculturing at 37° C. on slants of Francis' cystine-glucose agar.[9] Yeast phase cells from a 5-day-old culture were suspended in 0.5 per cent formalized physiological saline and stored in the refrigerator for sixteen hours. Following three washings with physiological saline the cells were resuspended in 0.1 per cent formalized saline and adjusted to the No. 3 McFarland nephelometer standard. Pairs of rabbits were immunized simultaneously with the formalin killed yeast phase cells of each strain of *H. capsulatum*. The rabbits received the following volumes of cells intravenously for six successive days: 0.1, 0.2, 0.5, 1.0, 2.0 and 2.0 ml. Two milliliter injections were then given on the seventh and fourteenth day following the last inoculation. All the rabbits were exsanguinated six days after the final injection. Breakage, however, resulted in loss of the A 228 rabbit antiserum.

Complement Fixation Procedure.—The antigenic titer of the mycelial filtrates was determined by means of the complement fixation test described by Tennenberg and Howell[3] in which serial dilutions of the antigens and antisera were tested in 0.2 ml. amounts, with the addition of 2 full units of complement, 2 units of hemolysin, and a 2 per cent suspension of sheep erythrocytes. The titers were recorded as the highest dilution of antigen which gave a three or four plus reaction with the smallest amount of serum.

In all, each of the 9 antisera was tested, wherever possible, against its homologous SC and SG antigen, as well as 7 heterologous single strain SC antigens, 8 heterologous single strain SG antigens, and 3 pooled preparations. One of the pooled lots was a mixture of equal amounts of the 8 single-strain histoplasmins from the SC medium, the second consisted of an equal amount of histoplasmin from 9 different strains of *H. capsulatum* grown on the SG medium, while the third pool designated as HF_1, was received from Dr. M. L. Furcolow. The number of strains used in preparing this last pooled antigen also grown on Smith's medium was unknown. Sera from 9 proved cases of human histoplasmosis were tested with each of the SC antigens, the pooled SC, and a single strain, HF_2 antigen, also received from Dr. Furcolow.

EXPERIMENTAL RESULTS

Before determining the comparative antigenic potency of each lot of histoplasmin, tests were conducted to ascertain whether complement fixation antibodies could be detected in the serum of each of the hyperimmunized rabbits and if significant differences in titer existed between the two rabbits immunized

TABLE I. COMPLEMENT FIXATION TITERS ON EACH PAIR OF RABBIT SERA TESTED AGAINST HOMOLOGOUS ANTIGENS FROM TWO MEDIA: SC AND SG

PAIRED

TABLE I. COMPLEMENT FIXATION TITERS ON EACH PAIR OF RABBIT SERA TESTED AGAINST HOMOLOGOUS ANTIGENS FROM TWO MEDIA: SC AND SG

PAIRED RABBIT SERA	SINGLE-STRAIN HISTOPLASMA CAPSULATUM ANTIGENS								
	28	99	105	208	232	A 233	A 235	267	430
1a	SC 64/256 SG 64/256								
1b	SC SG								
2a		SC 8/64 SG 4/4							
2b		SC 4/64 SG 0							
3a			SC 16/4096 SG 0						
3b			SC 8/16 SG 0						
4a				SC SG 32/4					
4b				SC SG 8/4					
5a					SC 32/2048 SG 32/16				
5b					SC 16/4096 SG 16/16				
6a						SC SG 16/128			
6b						SC SG 32/128			
7a							SC 64/2048 SG 64/128		
7b							SC 0 SG 0		
8a								SC 16/2048 SG 4/4	
8b								SC 16/1024 SG 4/4	
9a									SC 64/16 SG 64/256
9b									SC 32/8 SG 64/64

(Numerator designates highest serum dilution giving a 3 or 4 plus reaction with the highest antigen dilution [denominator])

208 SC flasks discarded due to contamination.

A 233 SC flasks discarded due to contamination.

Rabbit No. 1b. died.

Rabbit No. 7b. did not develop antibodies detectable with its homologous SC and SG antigens.

against the same lot of antigen. Accordingly, each serum was tested against its homologous antigen, grown on the SC and SG media. The highest dilutions of serums giving a three or four plus reaction as selected by a box titration with the highest dilutions of the homologous antigen are noted in Table I. The numerator represents the highest serum dilution, and the highest antigen dilution is designated in the denominator. It may be seen that with the exception of rabbit 7b, in whose serum no complement fixing antibodies could be detected, all sera showed some activity with either the SC or SG antigen. For use in the next series of tests that serum from each pair of rabbits showing the higher titer was selected.

The results obtained from the series of titrations of the various histoplasmin preparations against the rabbit antisera are presented in Table II.

TABLE II. COMPLEMENT FIXATION TITRATION OF HISTOPLASMA CAPSULATUM ANTIGENS AGAINST HOMOLOGOUS AND HETEROLOGOUS ANTISERA

ANTI-SERA	MEDIA	ANTIGENS											
		28	99	105†	208‡	A 228	232	A 233§	A 235	267	430	POOL	HF_1
28	SC	16384	128	4096		16384	4096		1024	1024	0	4096	8192
	SG	512	32		32	1024	32	1024	512	8	0	4096	
99*	SC	16384	128	2048			4096		512	256	0		
	SG												512
105	SC	8192	512	8192		8192	8192		4096	4096	0	8192	8192
	SG	4096	512		64	4096	1024	4096	2048	32	8	512	
208	SC	32768	256	8192		8192	8192		1024	1024	256	16384	2048
	SG	2048	32		16	2048	128	2048	1024	8	0	512	
232	SC	16384	1024	4096		8192	16384		2048	2048	256	8192	4096
	SG	1024	32		16	512	64	512	512	8	0	128	
A 233	SC	8192	256	2048		8192	8192		512	1024	64	4096	1024
	SG	512	8		8	256	32	256	128	0	0	128	
A 235	SC	32768	1024	8192		16384	16384		8192	2048	0	8192	4096
	SG	512	32		32	512	4	256	256	8	0	256	
267	SC	8192	128	4096		4096	2048		2048	1024	128	8192	2048
	SG	1024	64		32	2048	128	1024	1024	4	0	256	
430	SC	4096	256	128		1024	1024		64	64	256		2048
	SG	32	0		0	16	0	8	8	0	512	64	

*99SG Insufficient serum to run SG titration.
†105 SG antigen unusable through contamination.
‡208 SC antigen unusable through contamination.
§A 233 SC antigen unusable through contamination.

The figures cited represent the highest dilution of antigen that gave 75 per cent or more inhibition of hemolysis with the highest dilution of antiserum. Significant antigenic differences were found to exist among the tested strains of *H. capsulatum*. By inspection it was noted that strains 28 and A 228 produced the most reactive antigens with respect to the test sera on both the SC and SG media. Strains 232, A 235, and 233 were next in reactivity, but varied with the media used. Strains 99, 267, and 430 produced the least reactive antigens on both media. Data from strains A 233, 105, and 208 were not available for comparison on both media. The antigens studied may be placed in

the following order of descending activity: 28, A 228, pool, A 235, 232, 99, 267, and 430. The antigens produced in the SC medium rather consistently gave higher titers than those obtained from the SG medium. In only three instances were the titers equal. Pooling of the SC and SG antigens generally gave lower values than those obtained with the most active components of each pool. The pooled HF_1 antigen was approximately equal in reactivity to the pooled SC antigen.

Following these tests with hyperimmune rabbit sera, a group of 9 positive human sera from proved cases of histoplasmosis was tested with the single strain SC antigens, the pooled SC and SG antigens, and the single strain HF_2 antigen, which was then being used routinely in the C. D. C. serology laboratory for diagnostic work. The SG single strain antigens were not used in this phase of the study due to their low reactivity. The relative sensitivities of the antigens were determined with 4 units of each positive human serum. These units were calculated from the original titers of the sera obtained with a 1:10 dilution of the HF_1 antigen. The results of these tests are presented in Table III. As found with the rabbit sera, antigens from strains 28 and A 228 were

TABLE III. HISTOPLASMIN† TITRATION AGAINST FOUR UNITS OF HUMAN SERUM FROM PROVED CASES OF HISTOPLASMOSIS

SERA \ ANTIGENS	28	99	105	A 228	232	A 235	267	430	POOL SC	POOL SG	HF_2
1*	128	16	32	256	64	32	4	8	64	16	16
2	64	16	16	128	8	0	0	4	32	8	0
3*	128	16	16	256	16	8	4	4	16	16	16
4	128	0	4	256	32	16	0	4	64	8	16
5	128	16	16	256	32	16	4	8	64	16	8
6	128	16	16	128	16	4	0	8	64	8	4
7*	256	8	16	512	128	32	16	16	64	8	32
8*	128	16	16	256	16	4	0	8	64	32	8
9	128	32	32	8	16	32	256	64	64	32	16

*Starred sera received from Dr. M. Furcolow, all others from the CDC Serology Laboratory.

†Individual SC, pooled SC, SG, and a single strain antigen designated as HF_2 used routinely in the Serology Laboratory.

more reactive than the other SC antigens, the HF_2 antigen, or the pooled SC and SG antigens.

DISCUSSION

The results of this study suggest that the reactivity of the histoplasmin antigens elaborated under the conditions of the experiment varied with the strain of *H. capsulatum* used as the inoculum. This was not unanticipated, for it is well established that organisms within a species vary considerably in genetic constitution and consequently in physiologic expression. This finding, however, was contrary to the experience of Salvin and Hottle[5] who found no significant differences in the antigenicity of histoplasmin produced by 7 of 8 strains of *H. capsulatum* and attributed the failure of the eighth strain to produce a high titered antigen to its slow rate of growth. In our experiments

excellent growth of the fungus in all of the culture flasks would indicate that poor reactivity was not a consequence of poor growth.

The antisera titers from homologous and heterologous antigens were not significantly different. These data would indicate that homologous antigens are not necessarily superior to heterologous ones in demonstrating antibody titers in sera under the conditions of the experiment in which only hyperimmunized rabbits were tested.

Pooling of antigens served only to dilute the most potent components of the mixture. This was brought out in tests with rabbit antisera and with human sera obtained from proved cases of histoplasmosis, where pooled antigens from two types of media were consistently less sensitive than the best single strain antigens, with the exception of the reaction obtained with serum No. 9. This anomaly indicates the possibility that a pool of several highly potent strains might more consistently detect positive sera. The reported ability of a glycogen medium to produce highly antigenic lots of histoplasmin in short periods of time (Salvin and Hottle[5]) was not substantiated in the present experiment. The antigens produced in the SG medium in nine weeks (optimum time as determined by Salvin and Hottle) were in general less reactive than those obtained in the SC medium incubated for six months.

Due to the great length of time necessary to prepare the antigens and to study their serologic activity, data concerning the reproducibility of results are not available at this time. Whether certain strains will consistently produce highly reactive antigens over a period of years is under investigation.

SUMMARY

Ten single strain lots of histoplasmin were prepared to determine their relative antigenicity in complement fixation tests. Differences in potency were noted among them.

Pooled lots of histoplasmin were found to be less reactive than the highest titered components. A method for producing potent histoplasmin antigens employing a glycogen medium and a relatively short incubation period was found to be inferior to an older method in which a different medium and a longer incubation period was utilized.

Tests with sera from proved cases of human histoplasmosis with the exception of antigen A 228 on serum number 9 confirmed the results obtained with rabbit sera.

This study emphasizes the fact that histoplasmin is a crude antigen, subject to wide variations in potency, and that care must be exercised to select lots of high titer for use in histoplasmosis complement fixation tests.

Grateful acknowledgment is given Dr. M. L. Furcolow, Senior Surgeon, U. S. Public Health Service, Communicable Disease Center, Kansas City, Kan., for kindly furnishing 2 antigen preparations, designated as HF_1 and HF_2, and 4 sera from proved cases of human histoplasmosis.

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AN EVALUATION OF THE MIDDLEBROOK-DUBOS HEMAGGLUTINATION TEST

EDWARD F. RABE, M.D.,* AND WILLIAM S. SPICER, JR., M.D.**
WITH THE TECHNICAL ASSISTANCE OF MARGARET CHAPIN
KANSAS CITY, KAN.

THE MIDDLEBROOK-DUBOS hemagglutination test is a serologic technique of potential value to persons interested in tuberculosis. In the test, as originally described,[1] sheep red blood cells sensitized with a saline extract of mammalian tubercle bacilli were agglutinated by sera of rabbits immunized against BCG and by sera of human beings infected with tuberculosis. Red blood cells so sensitized exhibited an unusual serological specificity in the agglutination reaction. Appropriate tests with an agglutination-inhibition system indicated that the red blood cell sensitizing fraction of the tubercle bacillus was a polysaccharide.

Scott and Smith[2] modified the original technique of the test. These investigators used a commercially prepared concentrated solution of old tuberculin (Lederle) as the red blood cell sensitizing material. This antigen has since been used widely in the hemagglutination test reported by other workers.

Published reports upon the Middlebrook-Dubos hemagglutination reaction[1-7] have concluded with divergent views on the specificity and sensitivity of the test. In only one report[3] was the reproducibility of the test mentioned. The purpose of this communication is to evaluate the reproducibility, sensitivity, and specificity of the test when old tuberculin sensitized sheep red blood cells are used as antigen. The effect of intracutaneous P.P.D. upon the serological titers measured by the test is also described. The study is based upon the results of tests made upon 372 sera obtained from 199 individuals.

METHODS AND MATERIALS

Sera were tested in groups of 10 using an adaptation of the procedure described by Scott and Smith[2] and by Rothbard and associates.[6] Eagle's buffered saline† was used exclusively throughout the test. Inactivated sera diluted 1:2 were absorbed with washed packed sheep's cells at room temperature. The sera were then stored at –20° C. until the tests were performed.

From the Departments of Pediatrics and Internal Medicine and the Hixon Memorial Laboratory, University of Kansas School of Medicine.

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*Department of Pediatrics.

**Department of Internal Medicine.

†Eagle's buffered saline is prepared as follows: sodium chloride, 170 Gm.; KH_2PO_4, 2.7 Gm.; Na_2HPO_4 (anhydrous), 11.3 Gm.; add distilled water to make one liter. Dilute 1:20 with distilled water for use. This yields a final pH of 6.8 to 7.2. This solution is referred to as "buffered saline" subsequently in the test.

The antigen was prepared by adsorbing Lederle's old tuberculin concentrated four times the international standard* upon washed packed sheep's red blood cells. Lederle's old tuberculin was diluted 1:15 with buffered saline prior to its use as the red blood cell sensitizing agent. The sensitized cells after washing were placed in sufficient buffered saline to give a 0.2 per cent suspension. This suspension was used as antigen on the day of preparation. A similar suspension (0.2 per cent) of unsensitized washed sheep's red blood cells in buffered saline was prepared for use as a control antigen.

In each test consisting of 10 sera the following controls were included: (1) each unknown serum in dilution of 1:4, plus untreated sheep's cells in 0.2 per cent suspension, (2) buffered saline plus a 0.2 per cent suspension of sensitized sheep's cells, (3) a known high titered human serum plus a 0.2 per cent suspension of treated sheep's cells, (4) a known negative human serum plus a 0.2 per cent suspension of treated sheep's cells.

Initially much difficulty was experienced in reading the end point of the tests. The following procedure satisfactorily alleviated the problem. A constant indirect light source reflected by a slanted mirror was used to determine end points of hemagglutination. One individual read the test results. In some instances separate readings were made by two individuals, and the results were comparable. The procedure of reading each tube was as follows: The configuration of the cell button was first noted. The tube was then gently agitated into suspension, and the pattern of dispersion of the rising cells was noted. In the positive tests the cells usually rose quickly and blossomed into smaller clumps, while in the negative tests the cells rose slowly in stringy swirls. The final definitive reading was made by holding the tube at an angle on the slanted mirror and noting the clumping of cells outlined through the edge of the suspension by reflected light. The clumping was graded as follows:

4+ — large clumps in clear menstruum
3+ — slightly smaller clumps in cloudy menstruum
2+ — definite medium clumps in cloudy menstruum
1+ — very fine clumps in cloudy menstruum
0 — no clumping

The first 50 tests were checked microscopically and were found to show consistently clumping in the tubes read as 2+ or more, but did not show clumping in the tubes read as 1+ or 0. The end point was, therefore, defined as the last tube in which there was a definite 2+ reading.

The results of the tests are expressed as final dilutions of serum in buffered saline; the quantity of cells added as antigen has been disregarded.

RESULTS

Reproducibility of the Test.—Table I summarizes the results of repeated tests carried out on eighteen sera. Repeated tests with serum No. 1370 indicate the reproducibility of results obtained by using two separate lots of red-cell sensitizing material. The test was reproducible within the limits of a one-tube variation when either the same or a different lot of concentrated old tuberculin was used as a red cell sensitizing agent.

Sensitivity of the Test.—To determine the sensitivity of the test, two groups of patients were chosen: a group with active tuberculosis and a group with no evidence of tuberculosis. There were thirty patients in the group with active tuberculosis. Each patient in this group was included on the basis of showing all the following clinical and laboratory criteria: positive intracu-

*We are indebted to H. D. Piersma, PhD. of Lederle Laboratories for the generous supply of four times concentrated old tuberculin.

taneous tuberculin test performed after the last serum was drawn; roentgenologic evidence of disease in the chest or bone; positive bacteriologic evidence of tuberculosis by culture and guinea pig inoculation*; and clinical evidence of active disease during the time the study was being conducted.† Included in this group were 24 cases of pulmonary disease, 3 cases of meningeal and pulmonary

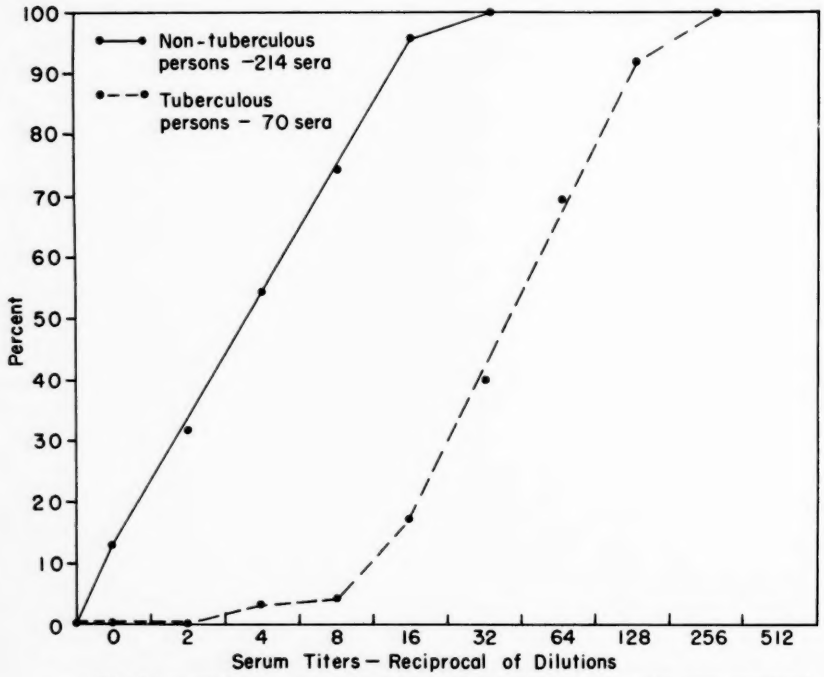

Chart I – Incidence, as accumulated percentages of the total sera examined, of hemagglutinin titers in two groups of patients.

disease, 2 cases of renal disease, and 1 case of osseus disease. Multiple serum samples were obtained from all but 10 patients. The minimum interval between samples was one month.

There were 81 persons in the group without tuberculosis. These persons included medical students, nurses, and college undergraduates. Each person was tuberculin tested (0.0001 mg. P.P.D. intracutaneously, read forty-eight hours after injection)‡ following the initial drawing of the blood. All were tuberculin negative. Four blood samples were taken from the nurses and medical students comprising the group; and only one sample was taken from the remaining 24 persons in the study.

*This indicates positive sputum in pulmonary disease, positive urine in renal disease, positive spinal fluid in meningeal disease, and positive bone aspiration in osseous disease.

†All patients save ten were under the observation of either one of the authors. We were kindly aided by Dr. Lawrence E. Woods. Ten patients were under the care of the Staff at the Veterans Administration Hospital, Excelsior Springs, Missouri, a Tuberculosis Sanitarium.

‡A positive reaction was indicated by a papule 5 mm. or more in diameter.

Charts 1 and 2 summarize the information derived from this phase of the investigation. Among nontuberculous patients there were no titers above 1:32. Four per cent of the sera had titers of 1:32 and 96 per cent of the titers were less than 1:32. Expressed in terms of persons studied, all had titers less than 1:64, 4.8 per cent had titers of 1:32, and 95.7 per cent had titers less than 1:32.

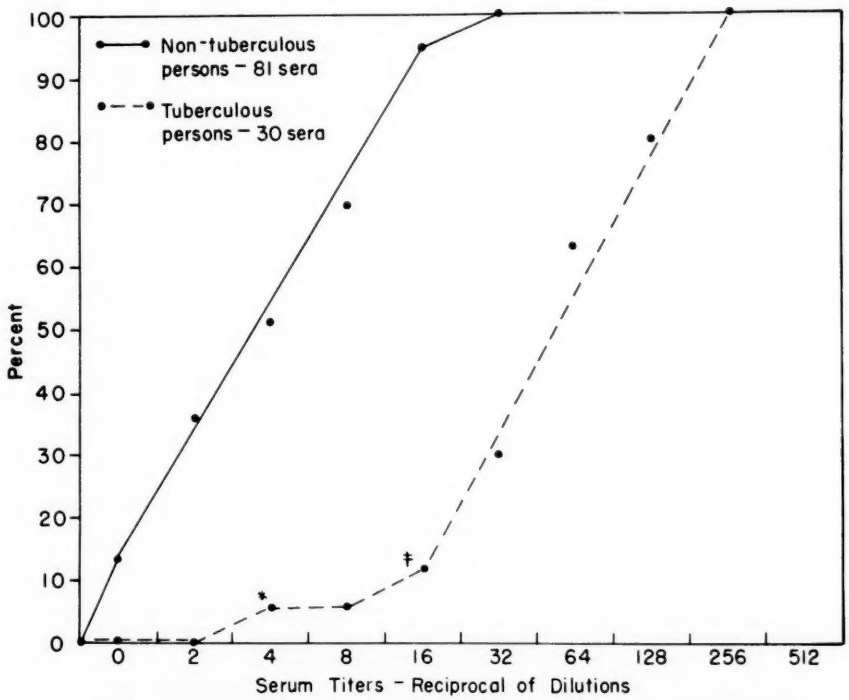

Chart II – Incidence, as accumulated percentages of total persons examined, of hemagglutinin titers in two groups of persons.
In persons having more than one serum examination, the highest titer obtained was charted for that individual.
* Both patients died shortly after sera were obtained.
‡ Both patients severely ill at time of sampling. One patient died shortly after sampling.

Patients with active tuberculosis exhibited titers of 1:64 or more in 60 per cent of the sera examined. Eight-seven per cent of the sera exhibited titers of 1:32 or more. Of the 12.9 per cent (9 samples) of sera with titers of 1:16 or lower, 4 sera were obtained from patients who were severely ill with tuberculosis. The remaining 5 low titered sera were obtained from patients who were being followed and from whom on earlier occasions sera with titers of 1:32 or greater were obtained. On a patient basis, 21, or 70.0 per cent, of the persons with active tuberculosis had titers of 1:64 or greater, and 26, or 86.7 per cent, had titers of 1:32 or greater at some time during their observed course of disease. There were 4 (13.3 per cent) patients with serum hemagglutination

TABLE I. HEMAGGLUTINATION TITERS IN REPEATED TESTS OF THE SAME SERA USING TWO LOTS OF ANTIGEN SENSITIZING MATERIAL

LOT OF O. T. 4× CONC.*	SERUM NUMBER	SERUM TITERS—RECIPROCAL OF DILUTIONS									
		0	2	4	8	16	32	64	128	256	512
2725-12	1449	3									
	1459	2									
	1586	2									
	1692	10									
	1108	1	1								
	1104					1	1				
	1105							2			
	1106								2		
	1107						2				
	1354							4	4		
	1625							2			
	1370						2	5			
2725-19A	1370						3	1			
	1398							2	2		
	1392							1	1		
	1755	3									
	1693	5									
	1694	2									

The numbers opposite the serum identification number indicate the frequency with which the titer, indicated by the heading of the vertical column, was obtained.

*Lederle's old tuberculin concentrated approximately four times the international standard.

titers less than 1:32, and each of these persons was extremely ill with tuberculosis.

A third group of persons was studied. This group included 23 individuals who were known to have been tuberculin positive. Each individual was clinically well and was working daily. Radiologic examinations of the chest had been performed on all persons. Most of the subjects had had serial chest roentgenograms and had evidenced no active pulmonary disease. Blood samples were drawn from these persons, and an intracutaneous tuberculin test was performed in the manner described above. Each of the persons proved to be a

TABLE II. HEMAGGLUTINATION TITERS IN PATIENTS WITH TUBERCULOSIS—ACTIVE DISEASE

PATIENT	STATUS OF PULMONARY TUBERCULOSIS	BACTERIOLOGY RE TUBERCLE BACILLI	ACTIVITY	HEMAGGLUTINATION TITER
H. R.	Bilateral cavitation. Febrile. Course downhill.	+ sputum	Active disease; died	1:4
W. M.	Far advanced, active. Febrile.	+ sputum	Active disease; died	1:4
W. T.	Miliary pulmonary disease. Cervical, inguinal, and femoral glands. Febrile.	+ sputum & nodes	Active disease; died	1:16
R. G.	Far advanced, cavitation both upper lobes.	+ sputum	Active disease	1:16
G. B.	Far advanced. Febrile, toxic.	+ sputum	Active disease	1:32
C. K.	Moderately advanced, active.	+ sputum	Active disease	1:32
E. H.	Progressive pulmonary disease.	+ sputum	Active disease	1:64
J. O.	Far advanced. Not regressing. Clinically well.	+ sputum	Active disease	1:64
T. S.	Far advanced. High fever.	+ sputum	Active disease	1:64
M. B.	Far advanced. Toxic, Pneumoperitoneum.	+ sputum	Active disease	1:256

TABLE III. HEMAGGLUTINATION TITERS IN PATIENTS WITH TUBERCULOSIS CONVALESCENT—ARRESTED DISEASE

PATIENT	PREVIOUS STATUS OF PULMONARY TUBERCULOSIS	BACTERIOLOGY RE TUBERCLE BACILLI IN SPUTUM	CLINICAL REMARKS	CONVALESCENT CATEGORY*	HEMAGGLUTINATION TITERS
R. J.	Moderately advanced	Neg. 13 mo.	—	4	0
D. W.	Moderately advanced	Neg. 14 mo.	Pneumoperitoneum	6	1:2
H. W.	Far advanced	Neg. 10 mo.	Pneumothorax, left	3	1:4
F. A.	Far advanced	Neg. 9 mo.	Right upper lobectomy	3	1:4
H. D.	Far advanced	Neg. 15 mo.	Thoracoplasty, right	5	1:4
W. D.	Minimal disease	Neg. 16 mo.	—	5	1:8
C. P.	Moderately advanced	Neg. 14 mo.	Pneumothorax, left	4	1:16
A. K.	Far advanced	Neg. 7 mo.	—	5	1:32
A. G.	Moderately advanced	Neg. 9 mo.	Thoracoplasty, right	6	1:32
T. L.	Far advanced	Neg. 12 mo.	Thoracoplasty, right	5	1:64

*Numbers refer to clinical category and indicate amount of activity permitted on an increasing scale, i.e., No. 1, positive sputum, complete bed rest; Nos. 2 to 6 negative sputum and increasing permissible activity; No. 7, negative sputum, released.

tuberculin reactor. The serum hemagglutination titers from this group ranged from 0 to 1:16, the greatest frequency of titers occurred at a level of 1:4.

The fourth group of patients, numbering 20, were hospitalized in a tuberculosis sanitarium. The patients were selected by the hospital staff so as to provide 10 examples of unequivocally active pulmonary disease, and 10 examples of pulmonary disease now in various stages of convalescence. One serum

TABLE IV. SERA USED TO TEST THE SPECIFICITY OF THE REACTION

DIAGNOSIS	NO. PATIENTS PER GROUP	SERUM TITERS RECIPROCAL OF DILUTIONS									NO. SERA PER GROUP
		0	2	4	8	16	32	64	128	256	
Healthy adults—Tbn. 0*	71	10	15	12	13	17	4				71
Histoplasmosis—Tbn. 0	5		2			3					5
Histoplasmin +—Tbn. 0 Fungus not recovered	5	1	1		1	2					5
Fatal granulomatosis* Tbn. 0	1	1									1
Healthy adults	8	1	1	4	1	1					8
URI Nonspecific	5	1		3	1						5
Streptococcosis	5	1		2	2						5
Carcinoma with lung metastases	2	1		1							2
Hodgkin's disease	1			1							1
Hypernephroma	1				1						1
Pregnancy	16		3	4	4	3	1	1			16
Syphilis	15		4	4	6		1				15
Total clinically nontuberculous	135	16	26	31	29	26	6	1			135

Serum hemagglutination titers in tuberculin negative persons and patients hospitalized for diseases other than tuberculosis. Numbers under serum titer columns indicate number of sera with each titer.

*All sera drawn before tuberculin test was performed.

specimen was obtained from each of the 20 patients. Hemagglutination titer determinations were performed without prior knowledge of the clinical condition of the subjects. Following the determinations laboratory and clinical correlations were made. These data are summarized in Tables II and III.

Specificity of the Test.—In Table IV data are presented which summarize the serum hemagglutination titers in tuberculin-negative healthy adults and in hospital patients admitted for conditions other than tuberculosis. In those patients listed as histoplasmosis, the fungus was recovered from the patient by culture, and complement-fixing antibodies were present in the patients' sera in significantly elevated titers*. Tuberculin testing (0.0001 mg. P.P.D. intracutaneously) was performed on four groups of patients, and all were tuberculin negative. In eight groups of patients tuberculin testing was not done. This fact is indicated by absence of comment on the tuberculin status.

Two pregnant women and one patient with syphilis had titers of 1:32 or greater. None of these 3 patients was available for tuberculin testing. In the total group there occurred 6 individuals (4.6 per cent) with serum hemagglutination titers of 1:32, and one (0.7 per cent) with a titer of 1:64. If tuberculin negative individuals (82 persons) only are tabulated, not one had a titer of 1:64 or greater, and 4 (4.9 per cent) had titers of 1:32.

Effect of P.P.D. upon the Serum Hemagglutination Titers.—A survey was conducted with the cooperation of a group of nurses and medical students to determine the effect of 0.0001 mg. of intracutaneous P.P.D. upon the serum hemagglutination titers.

The plan of study was the same for both groups. Blood was initially drawn. Tuberculin tests were performed as previously described. Blood was drawn one week, four weeks, and 8 weeks after the initial collection. In one instance, a patient with a highly positive tuberculin reaction, more frequent samples were drawn. Chest films were available on all subjects. These had been taken from a few days to approximately nine months prior to the survey.

Table V summarizes the results of the survey made on those 47 persons who were tuberculin negative. Each of those patients who responded sero-

TABLE V. EFFECT OF P.P.D. 0.0001 MG. INTRACUTANEOUSLY UPON THE HEMAGGLUTINATION TITERS OF TUBERCULIN-NEGATIVE PERSONS

	SERUM TITERS RECIPROCAL OF DILUTIONS						
	0*	2	4	8	16	32	64
Number of persons with initial serum titers as listed.	8	11	6	10	10	2	0
Number of persons showing a two-tube rise above initial titer—							
1 week after tbn. test	0	0	0	0	0	0	0
4 weeks after tbn. test	1†	1†	0	0	0	0	0
8 weeks after tbn. test	0	2†	0	0	0	0	0

*0 indicates absence of hemagglutination in the lowest serum dilution tested, i.e., ½.

†Figures indicate the initial hemagglutination titer. Example—initial hemagglutination titer, 2. If appearing in horzontal line "4 weeks after tbn. test" indicates hemagglutination titer at that time was 8.

*Sera for hemagglutination testing and data on the patient's skin test results, and complement-fixing antibody titers were kindly provided by Dr. M. L. Furculow of the United States Public Health Service.

logically to the injection of intracutaneous P.P.D., as judged by a fourfold rise in titer in paired sera, had initially very low or no demonstrable titers for hemagglutinating antibodies. None of the persons with initial titers ranging from 1:4 to 1:32 evidenced any serologic response to injected P.P.D.

Table VI shows the results of hemagglutination titer determinations on five patients with positive tuberculin reactions. Three of the 5 individuals responded to the tuberculin test serologically by titer increments of more than two tubes.

TABLE VI. EFFECT OF P.P.D. 0.0001 MG. INTRACUTANEOUSLY UPON THE HEMAGGLUTINATION TITERS OF TUBERCULIN POSITIVE PERSONS

PATIENT	AGE (YEARS)	TUBERCULIN REACTION*	SERUM TITERS—RECIPROCAL OF DILUTIONS INTERVAL AFTER TUBERCULIN INJECTION									SIGNIFICANT TITER CHANGE
			0	1 WK.	3 WK.	4 WK.	5 WK.	6 WK.	7 WK.	8 WK.	10 WK.	
J. S.†	5	+	0	64								+
H. H.	31	+	8	8		16				16		0
P. B.	26	+	16	16		32				32	16	0
R. H.	24	+	4	8		32				32	32	+
R. W.	28	+, ulcer formed	4	16	64	32	32	16	32	16	16	+

*A positive tuberculin was indicated by an area of induration 5 mm. or more in diameter, read forty-eight hours after the intracutaneous injection of 0.0001 mg. per 0.1 ml. of P.P.D.
†J. S. is a child who was not a member of the original survey group.

DISCUSSION

In determining the sensitivity of a serologic test, the ideal criterion is to show a significant titer difference between acute and convalescent serum specimens from the same person. Because of the long clinical course of tuberculosis, such a criterion is not quickly established. A second criterion is to determine a serum antibody level above which disease is to be found and below which no disease is to be found in the sampled patients. A titer of 1:64 or greater was invariably found in association with actively infected patients, and was never recorded in patients without evidence of disease. If a titer of 1:64 or greater is determined to be "significantly elevated," 30 per cent of tuberculous patients and 40 per cent of the serum samples from these patients are excluded from significance. If 1:32 is taken as a "significantly elevated titer," 13 per cent of tuberculous patients will be excluded and 4 per cent of nontuberculous persons will be included by the definition. A titer of 1:32 may therefore be considered of doubtful significance and 1:64 of definite significance in indicating active tuberculosis in the sampled patients. The converse of this statement is obviously not true. Patients with titers lower than 1:32 may be actively infected with tuberculosis and those with very low titers may even be dying of the disease. The theoretical aspects of this phenomenon have already been recognized and discussed by Middlebrook.[5] Although these conclusions may be of help in the diagnosis of active tuberculosis, they indicate the necessity for attempting to improve the sensitivity of the test. Such an improvement may be present in the complement-fixation test of Maillard.[8]

A third method of investigating the sensitivity of a serologic test is to determine the variations in titers between two groups of infected persons, those

with active disease, and those convalescent from tuberculous disease. The range in titer of a group with active disease was from 1:4 to 1:256 (average 1:55); the range in titer in the convalescent group was from 0 to 1:64 (average 1:16). The large overlap in titers from the two groups suggests a serious limitation of the test. Serial measurements of serum titers in convalescent patients over a long period of time may show the test to be more sensitive. Such a conclusion is suggested by the low average titer (1:5) found in 23 clinically well tuberculin-positive persons. However, since low hemagglutination titers can be associated with no disease or severe disease, the use of the serum titer alone as a measure of clinical activity of tuberculosis does not at present seem reliable.

The tuberculin test using P.P.D. as the antigen affects the serum hemagglutination titers. A significant titer change between two sera drawn at different times from the same person is defined as a fourfold or greater rise in titer. Titer changes of this magnitude were noted both in sera from patients who were tuberculin positive or tuberculin negative. The frequency and magnitude of these titer changes were not predictable. On the other hand, significantly elevated titers (i.e., 1:64) could be induced by intracutaneous P.P.D. only in tuberculin positive persons.

SUMMARY

The Scott and Smith modification of the Middlebrook-Dubos hemagglutination test was evaluated by a study of 372 sera from 199 individuals. The test was highly reproducible when conducted by the method described.

By defining a significantly elevated titer as 1:64, the test was sensitive enough to indicate clinical infection in 70 per cent of 30 tuberculous patients. A titer of 1:32 ("doubtful significance") was found in 87 per cent of tuberculous patients and 4 per cent of nontuberculous persons. Very low titers were found in tuberculous patients dying of their disease and in healthy persons.

The test was acceptably specific for tuberculosis on the basis of a "significantly elevated" titer being 1:64.

Intracutaneous P.P.D. caused significant titer changes in patients with positive or negative cutaneous reactions. Titer rises to 1:64 were observed only in patients with positive cutaneous reactions.

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THE MIGRATION AND OXIDATION-REDUCTION ACTIVITY OF LEUKOCYTES FROM PATIENTS WITH ACUTE DISSEMINATED LUPUS ERYTHEMATOSUS

S. N. Chaudhuri,* M.B.B.S., and Samuel P. Martin,** M.D.
Durham, N. C.

INTRODUCTION

THERE are series of morphological alterations induced by the exposure of blood and marrow cells to the plasma of patients with acute disseminated lupus erythematosus. One of these changes consists of the formation of the lupus cell.[1] The present study was undertaken in an attempt to correlate the morphological findings with alterations in cell function and metabolism. The migratory and the redox activity† of normal human leukocytes were compared with those from patients with acute disseminated lupus erythematosus. Observations were made on the redox activity of the leucocytes in vitro after exposure to autologous plasma and after the white cells of healthy subjects were exposed to plasma from the acute phase of the disease. The effect of adrenocorticotrophic hormone therapy on the behavior of the leukocytes was studied.

METHOD

Blood was drawn from the cubital vein into siliconed syringes and maintained in a non-wetting system. Heparin was used in order to prevent coagulation. For the studies on migration 0.0004 mg. per milliliter was used, and the blood was kept at 10 to 12° C. When the redox activity was measured, 0.04 mg. of heparin per milliliter was used, and chilling was unnecessary. The blood was handled throughout with siliconed pipettes and glassware. A modification of a method previously described was used in order to test the migratory activity[2] of leukocytes. The whole blood was added to a slide-cell constructed by two thin (3 mm.) layers of lucite bolted together with a rubber or plastic spacer (Fig. 1). The blood was added to the slide cell, which had been previously chilled to 10° C., the bolts tightened, and the cells sealed with petrolatum. The cells were centrifuged at a force of 200 Gm. for ten minutes. At the end of this time, the slides were placed in a vertical position in the incubator for fifteen minutes to allow clotting, then placed in the horizontal position, and the distance of migration was observed at six and twelve hours.

The leukocytes were separated from the red cells before study of their redox activity. Dextran‡ (intrinsic viscosity 0.46) was added to obtain a final concentration of 1.0 per cent. This induced rapid sedimentation of the red cells and allowed the separation of the white cells.[3] The white cells were separated from the plasma and the platelets by

From the Department of Medicine and Bacteriology, Duke University School of Medicine.
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*Permanent address: Indian Institute for Medical Research, Calcutta, India.
**Markle Scholar of Medicine.
†Redox activity is a chemical term used here to indicate mutual oxidation-reduction activity.
‡Supplied by Pharmacia Ltd., Park Ave., New York, N. Y. and Dr. James Tullis, University Laboratories of Physical Chemistry related to Medicine and Public Health, Harvard University.

centrifugation at 50 Gm. for seven and one-half minutes. The cells were resuspended in sufficient volume of plasma to obtain a count of 7,000 per cubic millimeter and exposed to the plasma for thirty minutes at 37° C. The number of cells present influenced the rate of reduction, therefore a constant cell count was maintained. Studies were carried out on cells from patients with acute disseminated lupus erythematosus suspended in autologous plasma. Cells from healthy individuals were also observed after exposure to the plasma of patients with acute disseminated lupus erythematosus.

A modification of Jensen and Maaloe's[4] method was used to measure the rate of reduction of the dye, 2-6 dichlorophenol-endophenol. The light absorption of the dye was measured at 600 mμ, and observations were made at zero, five, ten, twenty, and thirty minutes. This was done by measuring the optical density of the dye-leucocyte-mixture as compared with a mixture of the same number of leukocytes suspended in saline without the dye. Indication of this served as a standard for optical density of zero. The optimal concentration of 2-6 dichorophenol-endophenol was found to be 0.03 micromoles.

Studies were carried out on healthy medical students, patients with chronic diseases, and patients with acute disseminated lupus erythematosus. No attempt was made to survey a group of seriously ill patients; however, some are included in this series.

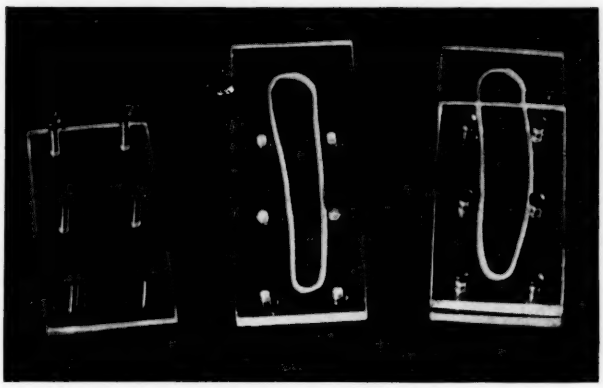

Fig. 1.—Photograph of slide cell. The cell on the right is completely assembled while the two parts are shown on the left.

RESULTS

There is marked impairment of migration of leukocytes in patients with acute disseminated lupus erythematosus. The mean six-hour migration of 10 healthy individuals was 2.0 ± 0.38 mm. per 6 hr., while that of 9 patients with acute disseminated lupus erythematosus is 0.7 ± 0.39 mm. ($p = < 0.01$). In addition to the changes in rate of migration in acute disseminated lupus erythematosus, there was clumping of leukocytes and hemolysis if the cells were observed at twelve hours. The migration returned toward normal after therapy with adrenal hormone in 6 patients followed for two weeks (1.5 ± 0.75 mm. per 6 hr.).

Fig. 2 shows the redox activity of leukocytes from 20 healthy subjects and 16 patients with acute disseminated lupus erythematosus. There is brisk conversion of the dye to the leukoform by the cells from the healthy subjects, but delayed and incomplete conversion in the 16 patients with acute disseminated lupus erythematosus.

Fig. 3 shows the redox activity of the leukocytes from two subjects with acute disseminated lupus erythematosus, and the effect of the plasma from these individuals on normal leukocytes. The plasma from patients with acute disseminated lupus erythematosus caused abnormal redox activity in leukocytes of the normal subjects. The plasma from subjects with acute disseminated lupus erythematosus lost the ability to alter the redox activity of normal leukocytes after several freezings and thawings or after storage at 10° C. for three days. This activity could not be restored by the addition of guinea pig complement. Fig. 3 also shows the values before and after therapy with ACTH. The upper two charts represent one subject before and after

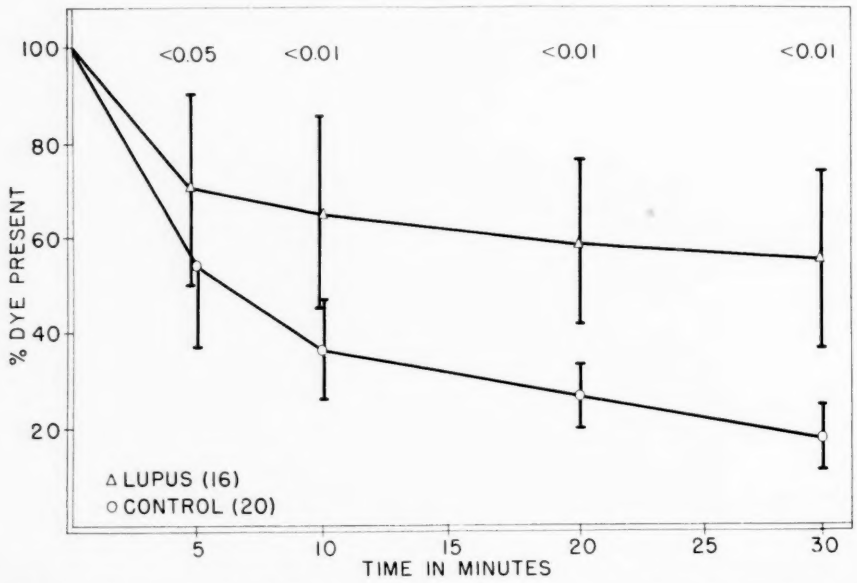

Fig. 2.—Redox activity of the leukocytes of 20 normal individuals and 16 patients with acute disseminated lupus erythematosus. The standard deviation of observations are indicated by the bars and the respective p values are noted above each time.

seven days of therapy. The lower charts show another subject after eight days of therapy. The redox activity returned to normal range after therapy. In both cases lupus cells were demonstrable at the time the redox activity had returned to normal.

In a series of 70 patients with other chronic diseases, 12 had an abnormal redox activity of the leukocytes and 7 had positive passive transfer tests. Five were patients with severe rheumatoid arthritis exhibiting pleural effusions and leukopena, but no lupus cells, 3 with questionable acute disseminated lupus erythematosus but no lupus cells, one with periarthritis, one with questionable rheumatic fever, one with malignant hypertension and one with severe anorexia nervosa.

There was marked diminution of redox activity of leukocytes exposed to antihuman-leukocyte-antisera* (Fig. 4). This antisera caused the cells of

*This serum was prepared by Dr. James Tullis, Univ. Laboratory of Physical Chemistry related to medicine and Public Health, Harvard Univ.

normal individuals to behave like those with acute disseminated lupus erythematosus (Fig. 3); however, it did not induce the morphological changes seen in lupus. The factor in this antisera was stable and persistent after treatment which would destroy the activity of sera of patients with acute disseminated lupus erythematosus.

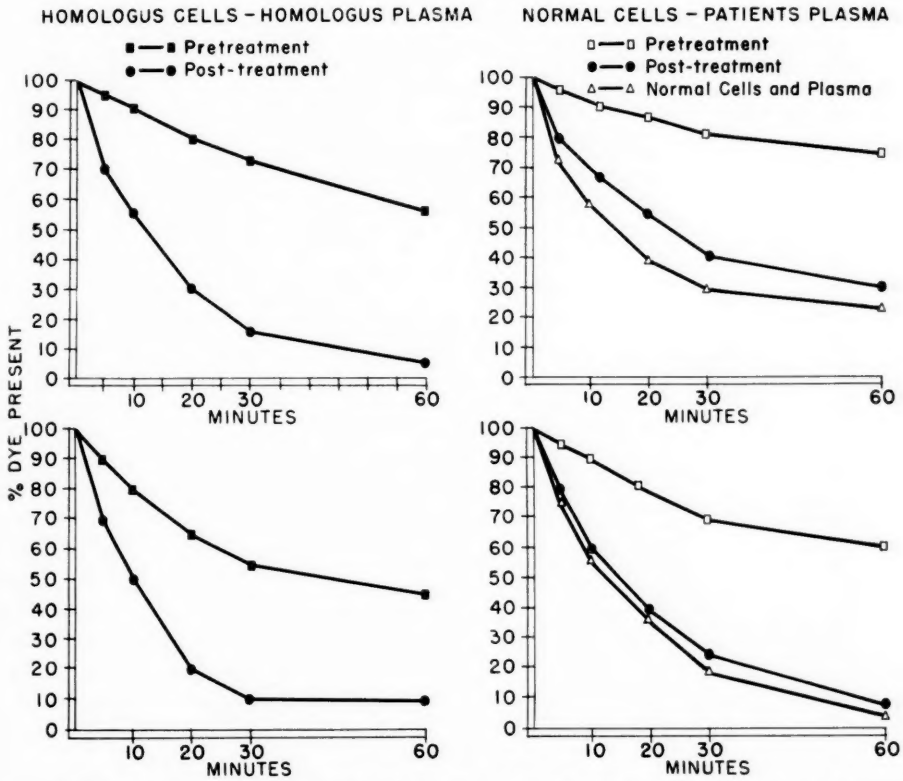

Fig. 3.—Charts of rodox activity of two patients with acute disseminated lupus erythematosus before and after adrenocorticotrophic hormone therapy. The upper two charts represent one patient. The chart on the left presents the data of homologus cells and plasma, and the right, normal cells with the patient's plasma.

DISCUSSION

Previous work has stressed the morphologic alterations in the leukocytes seen in patients with acute disseminated lupus erythematosus. The present work extends the observations pointing to a severe functional and metabolic change in these cells. There is impaired migration of the leukocytes in vitro. This may be on the basis of damage to the leukocyte induced by some of the known plasma factors that are found to be elevated or may reflect a defect in coagulation. The latter would cause a poor fiber network to support the migration of the cells. The clumping of cells noted on the slide may be a reflection of injury and may be on the same basis as the clumping noted when a whole series of toxic agents are added, as seen in the studies of Rebuck[5] and others on acute disseminated lupus erythematosus.

The changes in redox activity are marked, and reflect a profound alteration in the cell metabolic structure. This may come about by nonspecific means such as increase or decrease in cell membrane permeability or alteration in the redox system within the cell. In the present study there is an alteration in the rate, but also a significant alteration in the final redox potential. Jensen and Maaloe[4] have shown that the cell surface is the limiting factor in the rate of conversion of the dye. They have shown that low molecualr weight substances in the cell are responsible for this reduction. Glutathione and ascorbic acid will convert the dye to leukoform. It is interesting that Schoenbach and associates[6] have

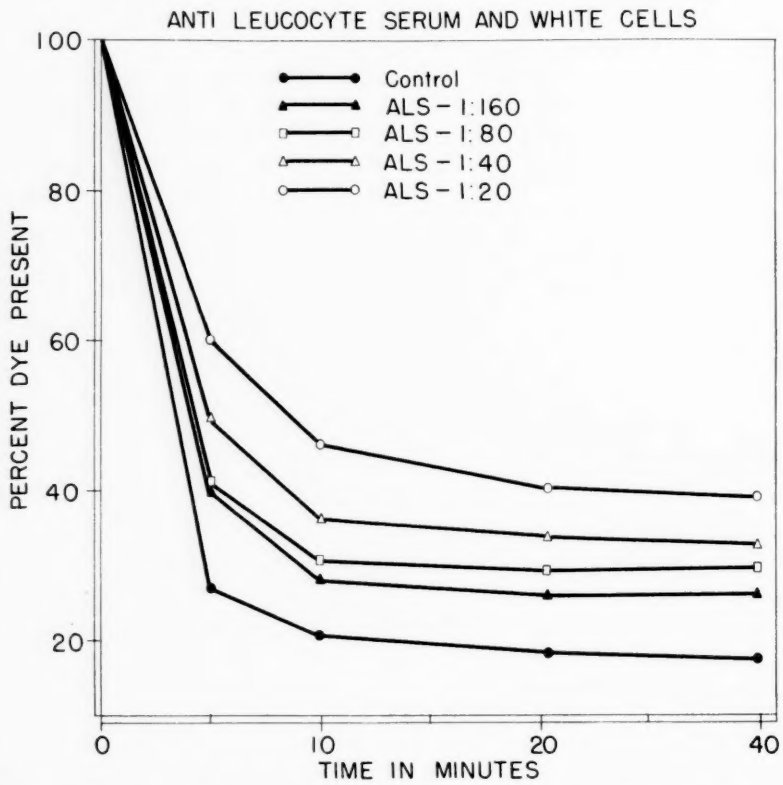

Fig. 4.—Redox activity of normal human leukocytes in the presence of dilutions of rabbit antihuman leukocyte antisera in various dilutions.

reported a low SH concentration in the plasma of patients with acute disseminated lupus erythematosus and would allow speculation as to the relationship to the present observations. At the present time, the site of the alterations is not certain.

The plasma factor responsible for the passive transfer does not appear to be the same as the stable globulin[7] which produces the lupus cell. The finding that this could not be restored by guinea pig complement shows that the lability was not due to complement loss which might have occurred with treatment.

Complement activity has been found to be low in acute disseminated lupus erythematosus.[8] Antileukocyte antisera, however, have the same reaction as plasma from patients with acute disseminated lupus erythematosus. This alteration in redox activity seen with antileukocyte antisera could serve as a basis for testing and confirming the thesis of anti-organ antibodies in a series of diseases, where they are expected, since the dye is reduced by brain and kidney slices. Further experimental steps will be necessary to determine the nature of this material in plasma of patients with acute disseminated lupus erythematosus.

SUMMARY

The leukocytes from patients with acute disseminated lupus erythematosus were studied and found to show marked alteration in the rate and character of migration in vitro. The cells also exhibited depression of redox activity. The plasma of patients induced abnormal redox activity in leucocytes from normal individuals after exposure for short periods of time.

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THE ROLE OF COLD HEMAGGLUTININS IN FROSTBITE

Capt. David Weiner, Medical Corps, Army of the United States
Fort Knox, Ky.

INTRODUCTION

LITTLE has been added to our understanding of cold hemagglutination since Landsteiner,[27] in 1903, gave the first adequate description of the phenomenon in guinea pigs, chickens, horses, dogs, and cattle. He demonstrated the dependence of the agglutination on low temperature with dispersion on rewarming, and was able to prepare solutions of agglutinin in normal saline solution. He further demonstrated that the agglutinin is contained in the globulin portion of the serum. The first description of the phenomenon in human beings was given by Biffi[5] in 1903. Clough and Richter[7] described the agglutinin as being in the euglobulin fraction, while Stats, Perlman, Bullowa, and Goodkind[41] demonstrated that it has the electrophoretic mobility of gamma globulin, and quantitatively determined its antibody nitrogen equivalence.

The serologic characteristics distinguishing cold hemagglutination from other types of agglutination have been well established and may be summarized as follows: (a) Agglutination of red cells by serum containing cold agglutinin can be demonstrated best between 0° and 5° C., rarely above 25° C., and practically never at 37° C. (b) The agglutination may be reversed by warming to 37° C., and reappears on cooling. This process may be repeated without apparent damage to the red cells. (c) Serum can be exhausted by repeated absorption in cold and released by rewarming. (d) The agglutinin is not inactivated by heating to 56° C. for thirty minutes. (e) The agglutinin survives cold storage with only slight decrease in strength for periods ranging from three weeks to a year or more. (f) The agglutinin is active against human cells of any group and against animal cells of many unrelated species.

The occurrence of cold hemagglutinins in normal healthy human beings has not been studied thoroughly. Freedman and Mirsky[15] found cold agglutinins in 14.6 per cent or 103 normal controls, Amzel and Hirzfeld[2] in 47 per cent of 238 sera obtained from routine bloods sent to the laboratory for Wassermann tests, and Kettel[25] in 95 per cent of 600 patients hospitalized for chronic diseases.

The association of cold hemagglutination with a variety of clinical conditions has been the subject of numerous reports. Comprehensive reviews of the literature were made by Stats and Wasserman[42] and by Platt and Ward.[33]

From the literature it appears that cold hemagglutination is found with a high degree of frequency only in primary atypical pneumonia[7, 12, 14, 15, 21, 32, 44] and in trypanosomiasis,[6, 10, 24, 47] in neither of which a pathogenetic relationship has been established. High titers are a constant feature in the former, reach-

From the Army Medical Research Laboratory, Fort Knox, Ky.
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ing their peak during the second to fourth week of the disease and falling rapidly during convalescence. The occurrence of cold agglutinins in malaria and other parasitic infestations has not been studied adequately.

The presence of cold hemagglutination in various types of acquired hemolytic anemias has been widely reported,[3, 9, 17, 20, 31, 37, 43, 45] but the diversity of hematologic pictures and the infrequency of occurrence of cold agglutinins make analysis of the relationship difficult. It is possible that cold agglutinins may play a pathogenetic role in certain types of hemolytic anemias, particularly those occurring during the course of primary atypical pneumonia.[14, 21, 32, 45]

Paroxysmal cold hemoglobinuria is not infrequently attributed to the presence of marked cold hemagglutination.[1, 3, 19, 26, 29, 34, 36, 40] Such cases can be differentiated clearly from the more common type of paroxysmal cold hemoglobinuria associated with syphilis in which the Donath Landsteiner test is positive and cold hemagglutination usually absent.[42] Raynaud's syndrome and similar peripheral vascular phenomena, with or without paroxysmal cold hemoglobinuria, have also been associated with cold hemagglutination.[1, 3, 4, 8, 9, 16, 18, 19, 22, 23, 29, 34, 38] Two such cases resulted in gangrene of the tips of the extremities.[29, 40] In the case reported by Stats and Bullowa[40] the mechanism was clearly defined by the demonstration of unilateral hemoglobinemia following exposure of one forearm to cold and by the demonstration of hemagglutination, discontinuity of the blood stream and slowing of blood flow in conjunctival vessels following irrigation of the sac with iced saline solution. Stats[39] further showed that cold-agglutinated red cells are easily hemolyzed by gentle shaking.

From the foregoing considerations, it is not too difficult to envision a relationship between the presence of cold hemagglutinins and the occurrence of frostbite and other forms of local cold injury. Such a thought, in fact, has not escaped recognition. Parker,[30] in a personal communication cited by Platt and Ward,[33] stated that cold-hemagglutination may play a part in the production of trenchfoot. Lange, Weiner, and Boyd[28] observed intravascular clumping of red cells in tissues exposed to cold, and suggested a relationship to cold-agglutinins or other agglutination reactions. Stats and Wasserman[42] suggested that individual tolerance to cold may depend on the titer of normal cold hemagglutinins.

In the present study an attempt is made to demonstrate a positive correlation between the occurrence of cold hemagglutination and the incidence of frostbite in military personnel.

MATERIAL AND METHODS

1. *Subjects.*—Cold agglutination tests were performed on 115 soldiers who had suffered frostbite of varying degree six to nine months previously during the 1950-1951 winter campaign in Korea. Tests were also performed on 308 unselected, apparently normal, healthy enlisted men from the Army Medical Research Laboratory and the Third Armored Division at Fort Knox. Approximately half of these men had seen combat service in Korea during the 1950-1951 winter. All of these men denied ever having suffered frostbite. The age and race distribution of frostbite and control subjects is shown in Table I.

TABLE I. AGE AND RACE OF FROSTBITE AND CONTROL SUBJECTS

AGE	FROSTBITE		CONTROL	
	WHITE	NEGRO	WHITE	NEGRO
17-20	33	16	34	49
21-24	25	17	72	52
25-30	9	6	41	16
31 or over	5	4	26	18
Totals	72	43	173	135

During the course of other experimental work in progress at the Army Medical Research Laboratory, three normal young enlisted men were repeatedly exposed to temperatures of –40° to –60° C. In two of these digital skin temperatures as low as –9° C. were reported, and first degree frostbite was often produced. Repeated cold agglutinin tests were performed on these men.

Cold agglutination studies were carried out on 13 rabbits before and after the production of experimental frostbite.

2. *Procedures.*—The method of determining the titer of cold agglutinin differed in no way from the methods reported in detail by nearly every investigator in the field. A few preliminary experiments were performed to confirm the fact that sera may be stored at 5° C. for a week or more without diminution in titer,[11, 12, 32, 33, 42] that cell suspensions from individual or pooled Group O donors and the subject's own cells (autoagglutinins) gave almost identical results, and that cell suspensions more than three days old were unsatisfactory. The procedures used throughout the present study are as follows:

(a) *Collection of blood specimens and preparation of sera:* Blood was collected in dry, sterile syringes from an antecubital vein, placed in 15 by 125 mm. test tubes and allowed to clot at 37° C. in a water bath or incubator. In a few instances clotting was permitted at room temperature (25° C.). After sufficient clot retraction had taken place the blood was centrifuged at 2,000 r.p.m. for at least thirty minutes, and the serum carefully decanted. In most cases the sera were tested immediately; in a few cases sera were stored at 0° to 5° C. for one to three days prior to titration.

(b) *Preparation of red cell suspensions:* In most instances cells from a single Group O donor were used. In a few instances pooled cells from several Group O donors were used. Cells were usually obtained by placing 2 to 5 ml. of freshly drawn blood in 50 to 100 ml. of warm, normal saline solution and separating by centrifugation. Occasionally, cells were obtained by washing the blood clot with warm saline solution. In either case the cells were washed at least three times by repeated centrifugation and resuspension in warm fresh saline solution. After having been washed, the cells were resuspended in sufficient saline solution to make a 1 per cent suspension.

(c) *Titration:* Serial dilutions of the serum by the doubling method were prepared in 12 by 100 mm. serologic tubes so that each tube contained 0.5 ml. of solution. To each tube 0.5 ml. of the red cell suspension was added, making the final serum dilution in the first tube 1:2, in the second tube 1:4, etc. The tubes were then shaken gently to assure even dispersion of the red cells and placed in a refrigerator at 0° to 5° C. The tubes were again gently shaken after being in the refrigerator thirty to sixty minutes, and immediately replaced to remain overnight.

(d) *Reading of agglutination:* After sixteen hours in the refrigerator at 0° to 5° C., the tubes were removed a few at a time and read immediately, before rewarming could occur. The tubes were shaken gently to loosen the red cell mass and observed with a two-inch magnifying lens in a moderately strong light. The agglutinations were graded in the following manner:

4 + massive agglutination of all or nearly all cells.
3 + large clumps with clear surrounding fluid.
2 + many smaller clumps with pinkness of surrounding fluid.
1 + minimal, but definite, agglutination visible.

The titer was reported as the highest dilution in which definite agglutination was visible. All agglutinations were confirmed to be cold agglutinations by observing disappearance on rewarming to 37° C.

RESULTS

1. Positive cold hemagglutination was present in 74.8 per cent of the frostbite cases and in 40.2 per cent of the normal controls. Titers, in general, were low as compared to those commonly found in primary atypical pneumonia, but ranged up to 1:64 in the frostbite group and 1:16 in the controls. The difference between the two groups is more strikingly apparent when the higher titers are considered. Titers of 1:8 or higher were found in 25.2 per cent of the frostbite cases and only 6.4 per cent of the controls. Table II summarizes the results obtained.

TABLE II. COLD HEMAGGLUTININ TITERS IN FROSTBITE AND NORMAL SUBJECTS

		FROSTBITE	CONTROLS
Total cases		115	308
Positive		86 (74.8%)	124 (40.2%)
Titers	1:2	41 (35.7%)	55 (17.9%)
	1:4	16 (13.9%)	49 (15.9%)
	1:8	16 (13.9%)	14 (4.5%)
	1:16	7 (6.1%)	6 (1.9%)
	1:32	4 (3.5%)	
	1:64	2 (1.7%)	
Negative		29 (25.2%)	184 (59.8%)

Analysis of these data by the chi square method and other methods indicates a statistical significance of better than 0.001. Thus, the probability of obtaining such results by pure chance is less than one in one thousand.

No difference in cold agglutinin titers was found between the control subjects who had fought in the Korean winter campaign of 1950-1951, and those who had not. Thus, of 162 Korean veterans 65, or 40.1 per cent had positive titers, and of the 146 others 59, or 40.4 per cent, were positive.

2. Positive cold agglutination occurred more frequently in Negroes than in whites both in the control group and in the frostbite group. Table III compares the results obtained in Negroes and in whites.

TABLE III. COLD AGGLUTININ TITERS BY RACE IN FROSTBITE AND CONTROL SUBJECTS

		FROSTBITE		CONTROLS	
		WHITE	NEGRO	WHITE	NEGRO
Totals		22	43	173	135
Positive		49 (68.0%)	37 (86.1%)	66 (32.8%)	58 (43.0%)
Titers	1:2	26 (36.1%)	15 (34.9%)	29 (16.8%)	26 (19.3%)
	1:4	10 (13.9%)	6 (13.9%)	29 (16.8%)	20 (14.8%)
	1:8	8 (11.1%)	8 (18.6%)	5 (2.9%)	9 (6.7%)
	1:16	4 (5.5%)	3 (7.0%)	3 (1.7%)	3 (2.2%)
	1:32	1 (1.4%)	3 (7.0%)		
	1:64		2 (4.7%)		
Negative		23 (32.0%)	6 (13.9%)	107 (61.8%)	77 (57.0%)

3. Within the limited age range of this series, age seemed to be a definite factor in the occurrence of cold agglutinins; the frequency, in general, decreas-

TABLE IV. THE INFLUENCE OF AGE ON COLD AGGLUTININ TITERS (NORMAL SUBJECTS)

		17-20	21-24	25-30	31 OR OVER
Totals		83	124	57	44
Positive		41 (49.4%)	45 (36.3%)	25 (43.9%)	13 (29.6%)
Titers	1:2	22 (26.5%)	19 (15.3%)	8 (14.0%)	6 (13.7%)
	1:4	14 (16.9%)	17 (13.7%)	14 (24.6%)	4 (9.1%)
	1:8	3 (3.6%)	7 (5.7%)	3 (5.3%)	1 (2.3%)
	1:16	2 (2.4%)	2 (1.6%)		2 (4.5%)
Negative		42 (50.6%)	79 (63.7%)	32 (56.1%)	31 (70.4%)

ing with advancing age. Table IV shows the titers obtained at different ages in the control group.

In view of the slight difference in distribution found between the frostbite and control groups, therefore, Table V is presented to compare the cold agglutinin titers of frostbite and control subjects who were in the 17 to 24 year age range. The results are similar to those for the entire group.

TABLE V. COLD AGGLUTININ TITERS IN FROSTBITE AND CONTROL SUBJECTS 17 TO 24 YEARS OLD

		FROSTBITE	CONTROL
Total cases		91	207
Positive		70 (76.9%)	86 (41.5%)
Titers	1:2	38 (41.8%)	41 (19.8%)
	1:4	11 (12.1%)	31 (15.0%)
	1:8	12 (13.2%)	10 (4.8%)
	1:16	5 (5.5%)	4 (1.9%)
	1:32	4 (4.4%)	0
	1:64	2 (2.2%)	0
Negative		21 (23.1%)	121 (58.5%)

4. In the three normal subjects exposed to experimental cold environment, cold agglutination tests were negative both before and after exposure.

5. In rabbits subjected to experimental frostbite cold agglutinin titers after exposure were practically identical to those obtained previous to exposure. Table VI shows titers obtained in 13 rabbits. Frostbite of varying degree occurred in all animals.

TABLE VI. COLD HEMAGGLUTININ TITERS IN RABBITS SUBJECTED TO EXPERIMENTAL FROSTBITE

RABBIT	DEGREE OF FROSTBITE	TITERS			
		BEFORE EXPOSURE	1 DAY AFTER EXPOSURE	8 DAYS AFTER EXPOSURE	15 DAYS AFTER EXPOSURE
B	First	1:8	1:16	1:16	1:8
C	First	1:8	1:8	1:16	
D	Third	1:16	1:16	1:16	
E	First	1:32	1:32	1:16	1:8
H	Fourth	1:64	1:32	1:16	
J	Fourth	1:32	1:32	1:16	
L	Fourth	1:16		1:8	1:8
M	Fourth	1:32		1:64	
N	Third	1:8	1:8	1:8	1:16
P	Fourth	1:16	1:16	1:16	
S	Fourth	1:16	1:16	1:16	1:8
W	Fourth	1:16	1:16	1:16	1:8
X	Fourth	1:16	1:16	1:16	1:16

DISCUSSION

There seems little doubt that a definite correlation exists between the occurrence of frostbite in exposed individuals and the presence of demonstrable cold hemagglutinins in the blood. This statement seems warranted in spite of the six- to nine-month interval between the frostbite expisode and the laboratory test, there being no reason to suppose that the control subjects did not have equal opportunities to acquire cold agglutinins during this period. The incidence of primary atypical pneumonia and other diseases associated with cold agglutinins was negligible in both groups.

The pathogenetic relationship of cold agglutinins to frostbite, on the other hand, is not conclusively proved. However, while the data are admittedly scant, the failure of appearance of cold agglutinins in the blood of the humans exposed to cold and the lack of rise of titer in the rabbits subjected to frostbite indicate that the reverse relationship does not exist. Thus, in an individual exposed to cold the presence of cold hemagglutination may cause intravascular clumping in the superficial vessels resulting in slowing of the blood flow and, perhaps, occlusion of small vessels. Intravascular clumping has actually been observed by Stats and Bullowa[40] and by Lange, Weiner, and Boyd,[28] in cold exposure.

It should be noted that the titers obtained in this study, for the most part, fall within the range considered "normal" by other investigators in the field.[2, 12, 13, 15, 33, 42, etc.] In the opinion of the author, however, this does not invalidate the finding that the incidence of frostbite may be significantly higher among individuals with "high normal" titers as compared to those with "low normal" titers. It must also be pointed out that minor differences in technique employed by the various laboratories apparently give rise to appreciable differences in results. Finally, it may be added that it has by no means been definitely established whether cold agglutinins are "normal" constituents of the blood or invariably the result of disease.

It is, of course, obvious that frostbite occurs in the absence of cold agglutination, since many individuals with high titers escape injury in spite of apparently severe exposure. This may be due to the multiplicity of factors involved in the production of clinical cold injury among which may be mentioned (1) degree of cold, (2) duration of exposure, (3) degree of moisture, (4) immobility, (5) nutritional status, (6) physical fatigue or exhaustion, and (7) the presence of peripheral vascular disease. Thus, when some or all of these factors combine to produce unusually severe conditions, the absence of cold agglutinins may not save the individual from irreversible injury. Conversely, under milder conditions an individual may escape injury in spite of the presence of marked cold agglutination. Conceivably, there may be a broad mid-zone of conditions under which the presence or absence of cold agglutination may play a decisive role.

SUMMARY AND CONCLUSIONS

The literature on cold hemagglutination is briefly reviewed.

A statistically significant relationship is found to exist between the oc-

currence of frostbite and the presence of demonstrable cold hemagglutinins in the blood.

Exposure to severe cold and first degree frostbite did not produce cold agglutinins in three human beings previously negative.

The titers of cold agglutinin did not increase in thirteen rabbits subjected to experimental frostbite.

From the available evidence, it is inferred that intravascular cold hemagglutination in an extremity exposed to cold may play a part in the pathogenesis of frostbite.

The more frequent occurrence of high titers of cold hemagglutinin in Negroes may be a factor in their reportedly[16] greater susceptibility to cold injury.

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ON THE DURATION OF PENICILLIN ACTION IN RELATION TO ITS CONCENTRATION IN THE SERUM

HARRY EAGLE, M.D., RALPH FLEISCHMAN, AND MINA LEVY, A.B., BETHESDA, MD.

UNDER appropriate conditions penicillin is directly bactericidal in vitro; and it is a reasonable presumption that its therapeutic action is based primarily on a similar direct effect. Host defense mechanisms may play an important role in supplementing that direct bactericidal action, but such host factors apparently do not significantly enhance the action of the drug as such. This is borne out by the following observations:

(a) The effective concentrations of penicillin for pneumococci, streptococci, and *T. pallidum* in vivo proved to be of the same order of magnitude as those effective in vitro,[1] and where not demonstrably affected by prior exposure to the drug.[2]

(b) The rate at which the bacteria were rendered nonviable in vivo was no greater,[2] and could be much less,[3] than that observed under optimal conditions in vitro.

(c) In mice, Type I and Type III pneumococci which had been exposed to penicillin were not abnormally susceptible to the host, and 1 to 10 viable organisms sufficed to cause a fatal infection. Penicillin-damaged Group A and Group B streptococci did, however, prove abnormally susceptible to the host, and until they had recovered from the toxic effects of the drug, the minimal infectious inoculum was temporarily increased ten to one hundred fold. [2]

(d) The time for which Group A streptococci, Type I pneumococci, or Type III pneumococci in a mouse muscle continued to die at a rapid rate after a single injection of aqueous penicillin closely approximated the time for which the drug remained at bactericidal levels in the serum.[2, 4] An exception was, however, noted with a Group B streptococcus in mice, which continued to die in a muscle focus for many hours after penicillin in detectable amounts had disappeared from the serum. This was not observed when the same organism was tested in rabbits.

(e) When mice were first injected with aqueous sodium penicillin, and at varying intervals thereafter challenged intramuscularly with a small number of Type III pneumococci, that small inoculum became capable of producing a fatal infection in more than half the animals as soon as the serum penicillin had fallen to an average level of 0.1 μg per milliliter.

(f) In a given infection, the curative dose (CD_{50}) on various schedules of treatment varied as much as one hundred fold, according to the frequency and duration of treatment; but at the CD_{50} level, all the dosage regimens provided effective concentrations of penicillin for essentially the same aggregate period,[4] unless the penicillin-free interval between injections had been so long as to permit the recovery and interim remultiplication of the surviving bacteria.

These data strongly suggest that the therapeutic action of penicillin is in large part measured by the aggregate time for which it remains at effective

From the Section on Experimental Therapeutics, Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health, Public Health Service, Federal Security Agency.

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levels at the focus of infection, that those effective levels in vivo are of the same order of magnitude as those effective in vitro, and that the effective penicillin time in the serum is usually a reasonable approximation of the penicillin time in the tissue fluids.*

There have been, however, several reports which suggest that the bactericidal action of penicillin regularly persists for significant periods of time after it is no longer demonstrable in the blood, either because the drug itself persists in the tissue fluids much longer than it does in the blood, or because the penicillin-damaged bacteria are disposed of by host defense mechanisms long after the drug itself is no longer operative. Thus, Ercoli and his co-workers[5] found that repository preparations of procaine penicillin in oil protected rats from infection with Type I pneumococci or Group A streptococci for four to eight hours after detectable penicillin had disappeared from the serum. Jawetz[6] also found that Group A streptococci continued to disappear from the blood and tissues of mice for as long as five hours after penicillin in the blood had fallen below the measurable levels of 0.03 units per milliliter. Recently, Weinstein, Daikos, and Perrin[7] demonstrated that if a fibrin clot was imbedded in the subcutaneous tissues of rabbits, and the animal was then injected with aqueous penicillin intramuscularly, the drug diffused into the clot, and there persisted for two to ten hours after it was no longer demonstrable in the serum.

In view of these discrepant results, experiments (d), (e) and (f) cited above have been repeated and extended. In the experiments to be here reported, there was again no evidence that penicillin persisted at effective levels in the normal skin, muscle, testis, or peritoneal cavity of mice or rabbits for significant periods of time after it had fallen below those levels in the serum. Further, although penicillin-damaged Group A streptococci sometimes continued to die for short periods after penicillin itself was no longer operative, that factor was quantitatively minor in relation to the preceding direct bactericidal action of the drug. Studies relating to point (f) above are discussed in a following paper.

EXPERIMENTAL

A. THE DIRECT MEASUREMENT OF THE BACTERICIDAL ACTION OF PENICILLIN IN VIVO AS A FUNCTION OF SERUM CONCENTRATION

Mice weighing 17.5 to 22.5 Gm. were inoculated intramuscularly into a hind leg with Group A streptococci (C-203 strain) in the logarithmic phase of growth. The inoculum of 4×10^6 bacteria, in a volume of 0.15 ml., was determined by dark-field microscopic count. Two hours later the animals were injected intramuscularly into the opposite leg with 0.15 ml. of sodium penicillin G in aqueous solution, at dosages of 0.05, 0.2, 0.8, 3.2, 12.5 or 50 mg. per kilogram. At varying intervals thereafter (⅜, ¾, 1½, 3, 4½, 6, 9, 12, 15, 18 and 24 hours), groups of 8 to 12 mice were sacrificed, the inoculated muscle was removed aseptically, emulsified in a Waring blendor with 50 ml. of 1 per cent blood broth, and the number of viable organisms determined by plate counts.

*There is no evidence that penicillin in a biologically active (bactericidal) form is strongly bound, either to tissue cells or to dissolved components of the tissue fluids; and one would anticipate a rapid equilibration between serum and tissue fluids. Barring the complication introduced by the presence of an abscess or cavity, a densely fibrotic area with poor blood supply, or the barrier interposed in equilibration between, e.g., the serum and spinal fluid, the time for which the drug remains at effective levels in the tissue fluids should closely approximate the similar penicillin time in the serum.[4]

TABLE I. THE BACTERICIDAL ACTION OF PENICILLIN IN A STREPTOCOCCAL INFECTION OF MICE

DOSAGE MG./KG.	MILLIONS OF VIABLE ORGANISMS IN INOCULATED MUSCLE AT INDICATED TIME AFTER TREATMENT										
	0	3/8 HR.	3/4 HR.	1½ HR.	3 HR.	4½ HR.	6 HR.	9 HR.	12 HR.	15 HR.	24 HR.
50	27			0.06	0.0053		0.0047	0.011	0.77	1.36	48
	24.5			0.044	0.0049		0.0019	0.0084	0.14	0.89	38
	22			0.034	0.0039		0.0013	0.0048	0.084	0.40	26
	21			0.021	0.0036		0.0012	0.0029	0.074	0.37	16.5
	19			0.017	0.003		0.0011	0.0017	0.034	0.18	7.5
	14			0.017	0.0025		0.0005	0.0013	0.003	0.034	7.0
	10			0.015	0.0018			0.00065	0.0012	0.034	3.0
	10			0.013	0.0013			0.00025	0.0007	0.0021	0.9
	Geometric mean* 17.3			0.024	0.0030		0.0014	0.0022	0.022	0.129	10.1
	S.E. (×/÷)† 1.22			1.21	1.19		1.38	1.60	2.4	2.25	1.64
12.5	Range* 16.4-51.2 (4)			0.062-0.092 (8)	0.0042-0.148 (8)		0.0114-0.042 (8)	0.124-0.847 (7)	1.2-13.1 (8)	3.2-76 (8)	
	Geometric mean 27.9			0.074	0.0165		0.196	0.52	3.44	24.5	
	S.E. (×/÷) 1.33			1.05	1.56		1.17	1.32	1.35	1.48	
3.2	Range 12.8-23 (5)		0.42-1.92 (8)	0.062-0.176 (8)	0.029-0.770 (9)	0.064-1.28 (8)	0. 051-0.468 (10)	1.8-21 (9)	0.173-102 (10)		0.552-91 (6)
	Geometric mean 19.3		0.37	0.10	0.13	0.17	0.135	7.67	4.70		8.54
	S.E. (×/÷) 1.005		1.22	1.14	1.44	1.45	1.28	1.36	2.03		2.34

0.8									
Range	9.7-28		0.14-0.8	0.06-1.1	0.02-4.3	0.055-7.5	0.51-37	22-112	102-369
No. mice	(5)		(6)	(8)	(8)	(8)	(8)	(6)	(6)
Geometric mean	16		0.29	0.27	0.39	0.63	6.55	52.3	154
S.E. $\left(\begin{smallmatrix}\times\\ \div\end{smallmatrix}\right)$	1.24		1.33	1.44	1.96	1.85	1.76	1.3	1.24
0.2									
Range	0.48-28	1.7-7.8	1.04-3.0	0.40-3.2	1.04-9.4	0. 64-20.8	0.56-44.8	24-320	
No. mice	(6)	(8)	(8)	(8)	(7)	(8)	(8)	(8)	
Geometric mean	16.2	5.74	1.9	1.47	3.3	3.1	20	67	
S.E. $\left(\begin{smallmatrix}\times\\ \div\end{smallmatrix}\right)$	1.24	1.22	1.14	1.30	1.47	1.54	1.45	1.38	
0.05									
Range	18-43	15-48	3.2-42	16-51	19-106	67-204			
No. mice	(6)	(8)	(8)	(8)	(8)	(7)			
Geometric mean	29	23	20	32	41	107			
S.E. $\left(\begin{smallmatrix}\times\\ \div\end{smallmatrix}\right)$	1.16	1.16	1.38	1.16	1.24	1.15			

Mice were inoculated intramuscularly with 4 x 10^6 Group A streptococci (C-203). Two hours later, they were treated into the opposite leg with sodium penicillin G at the indicated dosage. At the indicated intervals, groups of mice were autopsied, the inoculated muscle removed aseptically and emulsified in a Waring blendor. The number of organisms surviving in the muscle was determined by plating out a measured aliquot. The counts given in the body of the table (*range* and *geometric mean*) are the total number of viable organisms in the muscle in millions. For purposes of illustration, the detailed experimental data are given for mice treated at 50 mg. per kilogram. For reasons of clarity, not all the values here shown are indicated in Fig. 1; and one point in the figure (50 mg. per kilogram at two and one-quarter hrs.) is not included in the table.

*Total number of bacteria in the muscle, in millions.

†These values are the antilogs of the standard error of the mean logarithm. The standard error of the mean logarithm was estimated as $\frac{\text{range of log counts}}{\text{number of animals}}$ (after Mantel[11]).

The results are summarized in Table I and Fig. 1. In the figure, the heavy portion of the curves indicates the average time for which the serum penicillin remains in excess of 0.05 μg per milliliter at the indicated dosage of penicillin. The dotted portions of the lines indicate the additional times for which the serum penicillin remains in excess of 0.02 μg per milliliter. The choice of these two levels was dictated by the following considerations. Group A streptococci are killed in vitro at essentially the maximum rate by a penicillin concentration of 0.024 μg. per milliliter, while 0.008 μg. per milliliter is slowly but definitely bactericidal for 99.99 per cent of the organisms.[8] Since approximately half the serum penicillin is bound to the serum protein,[9] to provide these concentrations in the tissue fluids would require serum concentrations of at least 0.048 and 0.016 μg. per milliliter; and these have been rounded off to 0.05 and 0.02 μg. per milliliter.* The method used in calculating the time for which the serum

TABLE II. CALCULATION OF THE TIME FOR WHICH THE SERUM PENICILLIN IN MICE REMAINS IN EXCESS OF 0.05 AND 0.02 μG PER MILLIMETER AFTER VARYING DOSAGES OF PENICILLIN

(1)	(2)	(3)		(4)	(5)	
PENICILLIN DOSAGE MG./KG.	MEDIAN LOG SERUM CONCENTRATION (μG/ML.) AT TIME T FOLLOWING INJECTION*	CALCULATED TIME IN HOURS (FROM COLUMN 2) FOR WHICH SERUM PENICILLIN CONCENTRATION REMAINED IN EXCESS OF		PENICILLIN DOSAGES USED IN PRESENT EXPERIMENTS (MG./KG.)	ESTIMATED TIME IN HOURS† FOR WHICH SERUM PENICILLIN CONCENTRATION REMAINED IN EXCESS OF	
		0.05 μG/ML.	0.02 μG/ML.		0.05 μG/ML.	0.02 μG/ML.
200	2.5174-0.8555t	4.46	4.93	50	2.68 ± 0.18	3.07 ± 0.14
60	1.9239-1.2067t	2.67	3.00	12.5	1.71 ± 0.09	2.05 ± 0.067
10	1.3063-1.5972t	1.63	1.88	3.2	1.11 ± 0.06	1.38 ± 0.04
3	0.6835-1.9352t	1.03	1.23	0.8	0.7 ± 0.05	0.88 ± 0.03
1	0.2600-1.8220t	0.86	1.08	0.2	0.45 ± 0.045	0.62 ± 0.025
0.6	-0.0539-1.7176t	0.73	0.96	0.05	-	0.41 ± 0.021
0.15	-0.8128-1.6501t	0.30	0.54			
0.05	-1.3014-1.1259t	0	0.35			

*From curves fitted to experimental data of Eagle and associates.[12]

†These estimates of penicillin-time provided by a given dosage were obtained from curves fitted to the calculated times of column 3. The procedure is described in a previous paper.[4] These fitted curves were

(1) for 0.05 μg/ml., log penicillin time = −0.1215 + 0.3236 log penicillin dosage.
(2) for 0.02 μg/ml., log penicillin time = −0.0146 + 0.2933 log penicillin dosage.

We are greatly indebted to Mr. Nathan Mantel of the Research Studies Section of the Office of Biometrics of the National Cancer Institute, who carried out these calculations.

penicillin remains in excess of these levels after a given dose of penicillin is summarized in Table II. As there shown, and as is evident in Fig. 1, except at barely effective dosages (on the order of 0.05 to 0.2 mg. per kilogram), it makes but little difference which threshold value is used in calculating the "penicillin time." This reflects the fact that in mice penicillin G disappears from the serum so rapidly that at dosages in excess of 0.2 mg. per kilogram, the time required for the serum penicillin concentration to fall from 0.05 to 0.02 μg per

*The latter value of 0.02 μg. per milliliter is considered a better estimate of the serum concentration which provides barely effective levels in the tissue fluids than the value of 0.012 μg. per milliliter in a preceding paper.[4] The latter would provide a maximum concentration of 0.006 μg. per milliliter in the tissue fluids; and although the Group A streptococcus here used is killed by this concentration in vitro, the bactericidal effect is extremely slow, with no demonstrable killing even after three hours, and is incomplete. As discussed in the text, because of the rapid rate at which penicillin disappears from the serum of mice after the injection of the sodium salt, the use of 0.02 instead of 0.012 μg. per milliliter as the threshold concentration has no signficant effect on the calculated "penicillin time."

milliliter is small in relation to the time for which it had previously remained in excess of 0.05 μg per milliliter.

Several points emerge clearly from this experiment: (1) The initial rate at which the streptococci were killed (heavy and dotted portions of curves, on left side of figure) was independent of the dosage and of the absolute magnitude of the serum concentration, provided only that the maximally effective concentration was provided at the focus of infection. In these experiments, as in those previously reported, and contrary to the suggestion of Schmidt and Walley[13], there was no indication that large dosages (e.g., 50 mg. per kilogram) were more rapidly bactericidal than small doses (e.g., 0.2 mg. per kilogram).

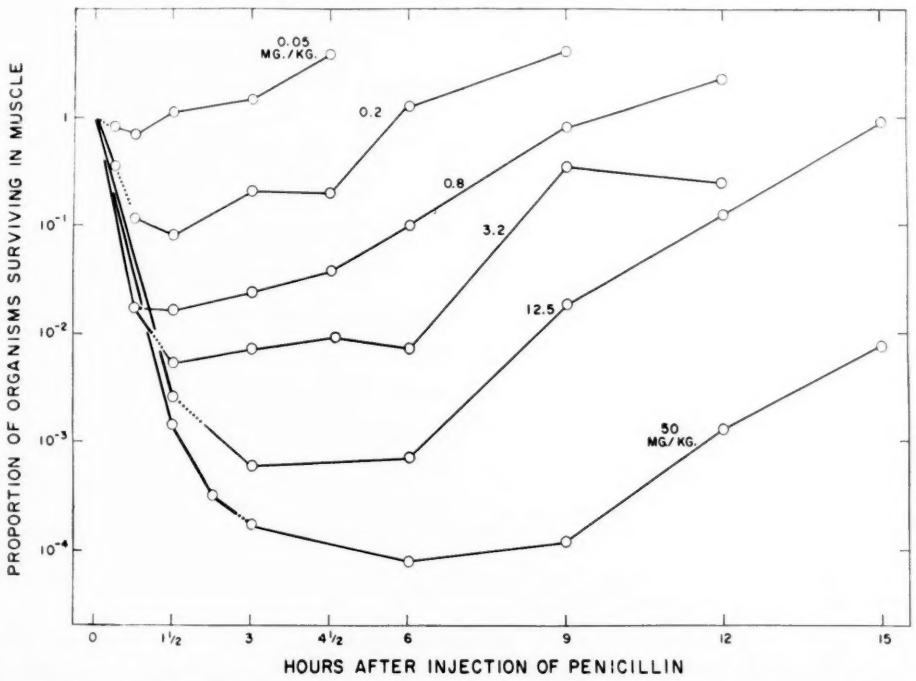

Fig. 1.—The bactericidal action of penicillin on Group A streptococci in a mouse muscle infection (after data of Table I). Each point in the figure is the (geometric) mean of the number of organisms surviving in a group of 6 to 10 mice, referred to the number present at the time of treatment as 1. The heavy line in the left-hand portion of each curve indicates the time for which the serum penicillin had remained in excess of 0.05 μg per milliliter; and the dotted portions of the curves indicate the additional time for which the serum level had remained in excess of 0.02 μg per milliliter. As discussed in the text, these two levels are estimated to provide the maximally and minimally effective levels in the tissue fluids.

This does not gainsay the possibility that in situations in which diffusion into the focus is a limiting factor (cf. footnote* on page 123), such large dosages may initiate the action of penicillin more rapidly by facilitating its diffusion into the focus of infection.

(2) The important difference between the varying dosages was not in the initial rate of the bactericidal action, but in its duration. The bacteria continued to disappear from the muscle at a rapid rate as long as the serum concentration of penicillin remained at levels sufficient to provide the effective con-

centration at the focus of infection. Whether mice were injected with penicillin at 50, 12.5, 0.8, or 0.2 mg. per kilogram, the organisms continued to die rapidly as long as the serum concentration remained in excess of 0.05 to 0.02 μg. per milliliter. As soon as the serum concentration fell below these levels, the bactericidal action was abruptly retarded. This is evident in Fig. 1. There was thus no indication in the present experiments that penicillin in effective concentration had persisted at the site of inoculation for a significantly longer time than in the serum, even after large doses of the drug.

(3) The duration of the following recovery period, during which the organisms were recovering from the toxic effects of the drug, varied widely in different animals (Table I and Fig. 1). It was of relatively brief duration after small doses, but averaged four to six hours at dosages of 3.2, 12.5, and 50 mg. per kilogram (Fig. 1). During this period, when the penicillin itself was no longer operative, host defenses supplemented the action of the drug to only a minor degree. The bacteria which had been damaged but not yet killed by the drug continued to die for only a brief period (averaging between zero and two hours in the several experimental groups of Fig. 1), and at a significantly reduced rate.

(4) When the organisms had recovered, multiplication was resumed, and the animals eventually succumbed to a generalized streptococcal infection.

B. THE DURATION OF THE PROTECTION AFFORDED BY A SINGLE DOSE OF AQUEOUS SODIUM PENICILLIN TO THE FOLLOWING INOCULATION OF STREPTOCOCCI OR PNEUMOCOCCI

A single large dose of aqueous penicillin (60 mg. per kilogram) was injected intramuscularly in mice. At varying intervals thereafter, groups of 20 to 55 mice each were challenged by the intramuscular, intraperitoneal, or subcutaneous injection of either Group A streptococci or Type III pneumococci. The inoculum was calibrated by dark-field microscopic count, and adjusted to contain 200 organisms. The virulence of the strains had been maintained by mouse

TABLE III. DURATION OF PROTECTIVE ACTION OF SODIUM PENICILLIN G IN MICE AFTER ITS INTRAMUSCULAR INJECTION AT 60 MG PER KILOGRAM

ORGANISM INOCULATED	ROUTE OF INOCULATION	PERCENTAGE MORTALITY IN MICE INOCULATED AT INDICATED TIME AFTER PENICILLIN							
		0*	1	1½	2	4	3	6	CONTROLS —NO PENICILLIN
Streptococcus agalactiae	intramuscular	5	0	15	90	100	100	100	100
Streptococcus pyogenes (C-203 strain)	intraperitoneal	0	0	2	11	40	69	98	63
	intramuscular	0	6	17	32	58	70	88	88
	subcutaneous		0	5	3	83	83	93	88
Diplococcus pneumoniae, type III	intraperitoneal	4	12	28	72	80	96	92	96
	intramuscular	0	5	40	75	85	100		95
	subcutaneous	0	6	30	60	85	100		
Median serum concentrations of penicillin at indicated time, μg./ml.†		-	3.3	0.95	0.25	0.046	<	<	-

Groups of 20-55 mice were challenged with 200 organisms at the indicated times after the injection of penicillin.

*Animals inoculated immediately after injection of penicillin.

†After Eagle and associates.[12] The value of 0.95 μg at one and one-half hours was obtained by graphic interpolation.

passage repeated two to three times weekly, and 1 to 10 organisms regularly produced a fatal infection in more than half the animals. The inoculum therefore represented on the order of 20 to 200 LD_{50}. Rabbits were similarly injected with penicillin and challenged by the intramuscular, subcutaneous, intraperitoneal, or intratesticular inoculation of a group B β-hemolytic streptococcus (*S. agalactiae*). The inoculum in this instance was 40 organisms, representing 4 to 40 LD_{50}. Small inocula were used in order that relatively small amounts of penicillin persisting in the tissues would nevertheless suffice to abort the infection.

The results are shown in Tables III and IV. Whether bacteria were inoculated intramuscularly, intraperitoneally, subcutaneously, or intratesticularly,

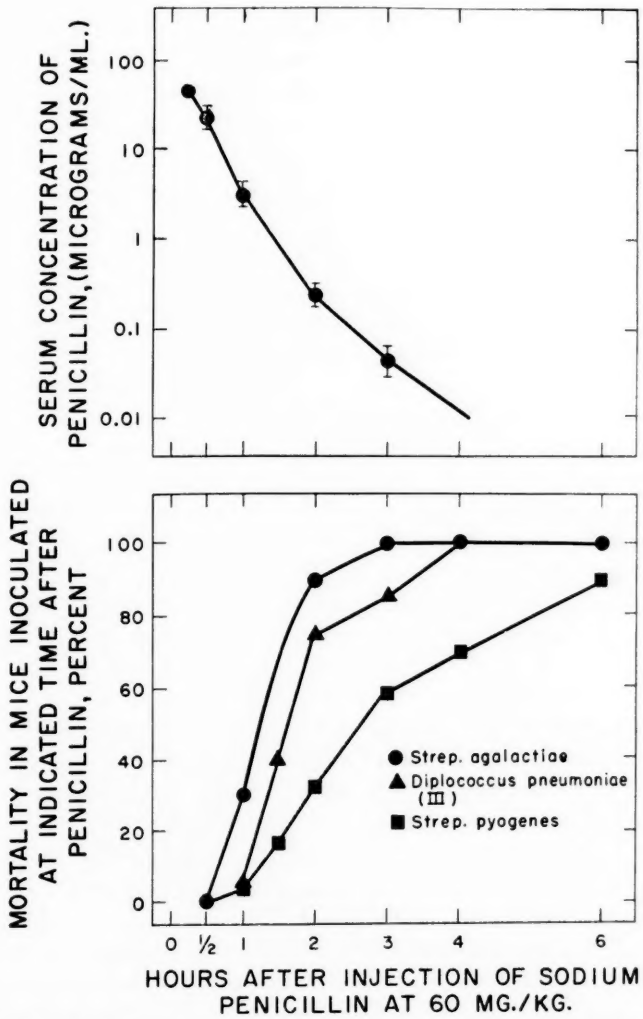

Fig. 2.—The duration of the protection afforded by a single injection of penicillin to the following inoculation of small numbers of virulent organisms (after data of Table III). The top curve shows the median serum concentration of penicillin (and its standard error) at varying intervals after the intramuscular injection of sodium penicillin G into mice at 60 mg. per kilogram.[12] The bottom curves show the percentage of mice which died when inoculated with 200 organisms at varying times after the injection of penicillin at this dosage level. The inoculum in each instance was 200 organisms, by microscopic count, and 20 to 25 animals were injected at each time period.

TABLE IV. DURATION OF PROTECTIVE ACTION OF SODIUM PENICILLIN G IN RABBITS AFTER ITS INTRAMUSCULAR INJECTION AT 60 MG. PER KILOGRAM

ROUTE OF INOCULATION	PROPORTION OF ANIMALS WHICH DIED WHEN INOCULATED AT INDICATED TIME (HOURS) AFTER PENICILLIN							CONTROL (NO PENICILLIN)
	1½	2	3	4	6	8	10	
Intramuscular	0/8	1/10	1/18	7/10	7/8	8/8		14/14
Intraperitoneal		0/6	0/8	0/8	2/8	3/8		7/9
Subcutaneous			1/8	1/10	8/10	10/10		12/12
Intratesticular		1/8	1/8	0/10	7/10	6/10	10/10	14/15
Median serum concentrations penicillin at indicated time, μg/ml.*	11	5.9	2	0.58	0.13	0.033	<	

Animals were challenged with 40 organisms (Streptococcus agalactiae) at varying times after penicillin.

*After Eagle and associates.[12] The values given for one and one-half and three hours were obtained by graphic interpolation.

the animals became susceptible to the challenge inoculum even *before* measurable concentrations of penicillin had disappeared from the serum. As illustrated in Fig. 2, a small challenge inoculum of Group B streptococcus, Type III pneumococcus, and a Group A streptococcus produced a fatal infection in half the animals if given 1.3, 1.6 and 2.6 hours after the penicillin, i.e., approximately 1.4, 1.1 and 0.1 hours *before* the serum penicillin concentration had fallen to an average level of 0.05 μg per milliliter. The latter values are a measure of the time required for penicillin to dispose of the challenge inoculum under the conditions of the present experiments. In vitro also, the Group A streptococcus was the most susceptible of the three species to penicillin in terms of both the effective concentration of penicillin and rate of its bactericidal action, while the Group B streptococcus was the least susceptible.[8]

Based on bactericidal action in vivo, there was no indication in these experiments that penicillin had persisted at effective levels in the tissue fluids of normal animals for a significantly longer period than in the serum.

DISCUSSION AND SUMMARY

The present experiments with mice and rabbits provide no evidence that penicillin persists at effective concentrations in the uninflamed skin, muscle, testis, or peritoneal cavity for a significant period after it has fallen below those levels in the serum. When the duration of penicillin action was tested by inoculating normal animals at varying intervals after a large intramuscular injection of penicillin, small challenge inocula proved infectious even before the serum concentration had fallen below 0.05 μg per milliliter.

The contrary results of Ercoli and his co-workers[5] could perhaps be explained on the basis that they used a repository preparation of penicillin rather than the sodium salt in aqueous solution. The serum concentration of penicillin then falls only slowly; and with highly sensitive organisms, which are killed by concentrations significantly lower than those measurable by a bio-assay procedure, the drug may be present in the serum and in the tissue fluids at effectively bactericidal levels for hours after it is no longer demonstrable in the serum. In the ex-

periments of Weinstein, Daikos and Perrin,[7] who demonstrated the persistence of penicillin in a fibrin clot for significant periods after it was no longer demonstrable in the plasma, the diffusion of penicillin into and out of a fibrin clot may be much slower than its equilibration between the tissue fluids and plasma in a normal capillary bed. The present experiments offer no evidence for the persistence of effective concentrations of penicillin in normal tissue fluids for significant periods after it has fallen below those levels in the plasma. The findings of Weinstein and his co-workers may, however, be of significance in relation to abscesses, cavities, or densely fibrotic areas into which penicillin may diffuse only slowly, and out of which it may then diffuse with equal slowness when the concentration in the plasma eventually falls below that in the tissue fluids.

In infected animals, and in confirmation of results previously reported,[2, 4] large doses of penicillin (e.g., 50 mg. per kilogram) were no more rapidly bactericidal than much smaller doses (0.2 mg. per kilogram) which sufficed to provide the maximally effective concentration. The advantage of the larger dose lay simply in the fact that effective concentrations of the drug, and thus its bactericidal action, were sustained for a longer period of time. The rapid bactericidal action of the drug in a muscle focus stopped as soon as the serum concentrations had fallen below the levels estimated to provide effective concentrations at the focus of infection.

The degree to which host factors enhance the direct bactericidal action of penicillin by continuing to dispose of penicillin-damaged bacteria after the drug itself has fallen to ineffective levels is debatable. In this laboratory the only indication of a marked effect had been obtained with an *S. agalactiae* infection in mice.[2] With either Type I or Type III pneumococcal infection, with Group A infections, or with the Group B infection in rabbits, the bacteria continued to die for only a brief period after penicillin itself was no longer operative, and at a greatly reduced rate. In the present experiments also, the rate of bactericidal action in an intramuscular focus of Group A streptococci decreased sharply as soon as the serum concentration of penicillin fell below the levels estimated to provide effective concentrations in the tissue fluids. Thereafter, the host defense mechanisms in these nonimmune animals disposed of the penicillin-damaged bacteria for a relatviely brief period, usually averaging less than one hour, and at a relatively slow rate as compared with the preceding bactericidal action of the drug.

These data support the thesis that in the nonimmune animal, the primary determinant of the therapeutic activity of penicillin is the total time for which it remains at the focus of infecion in concentrations effective against the particular organism.[2, 4, 10] The degree of bactericidal action accomplished by a given penicillin time will vary with the particular bacterial species, and is materially influenced also by the local conditions in the focus of infection.[3] Further, with too long an interval between successive injections, remultiplication of the surviving bacteria may vitiate in whole or in part the therapeutic effect of the preceding injection. In a given focus, however, the degree of bactericidal

action afforded by a single injection of sodium penicillin appears to be largely determined by the time for which it provides effective concentrations in that focus.

The extent to which the presence of antibodies modifies the therapeutic action of penicillin is under present study.

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CLINICAL LABORATORY STUDIES IN EXPERIMENTALLY-INDUCED EPIDEMIC NONBACTERIAL GASTROENTERITIS

IRVING GORDON, M.D., JOHN K. MENEELY, JR., M.D.,
GORDON D. CURRIE, M.D., AND ALICE CHICOINE
ALBANY, N. Y.

TRANSMISSION studies of epidemic gastroenteritis in volunteers have shown that one or more filtrable infectious agents are demonstrable in the feces of sick individuals.[1-5] Attempts to propagate a causative agent in experimental animals have failed thus far. To obtain fresh material for such experiments the Marcy strain of epidemic nonbacterial gastroenteritis[2, 4, 5] has been passaged several times in human volunteers. Advantage was taken of these experimental infections to characterize the disease further by means of clinical laboratory studies. All of the volunteers had previously been fed tissue culture specimens as a test for the presence of the inciting agent.

Investigation of the Marcy strain began in December, 1946, following an epidemic at the Marcy State Hospital, near Utica, N. Y. The outbreak was characteristic of others in New York and New England during that fall and winter. It lasted through January, 1947, involving 589 of the 2,623 inmates and 115 of the 354 employees. Epidemiologic studies indicated that it probably spread through person-to-person contact.[2, 4] In communities the unit of spread was the family.

The cardinal symptoms of infection with the Marcy agent were anorexia, nausea, vomiting, and diarrhea. These were often accompanied by abdominal cramps and borborygmi. Fever was low or absent. Experimental subjects had an incubation period of from one to five days, with a median of three days.[2, 4-7] Onset of acute illness was usually rapid, sometimes abrupt, although in a number of instances slight anorexia and hyperperistalsis presaged the development of the full syndrome. Both spontaneous and experimental cases sometimes failed to exhibit diarrhea, but this was exceptional; stools were usually frequent, profuse, and watery, without blood, pus, or mucus. The leukocyte count was normal except in dehydrated patients. The disease was mild; spontaneous recovery characteristically took place after a few days.

The infectious agent was transmissible in series by oral inoculation of fecal suspensions or filtrates from acutely ill patients. The experiments to be reported represent the seventh serial passage in volunteers of the Marcy strain. In the course of these passages 58 volunteers were fed varying doses of fecal inocula.[2, 4-7] Forty-two became ill. In contrast, none of seven subjects who inhaled throat washings from acutely ill patients developed the disease, while of ten who drank throat washings only two had symptoms, which were transient and of questionable significance. (The results of titrations of filtered stool or throat washings are excluded from these data because of the small doses employed.)

A total of eighteen recovered subjects were reinoculated orally with various infectious fecal suspensions within a few weeks after the original inoculation.[2, 4-7] All remained asymptomatic, showing that an attack of the disease confers an immunity of short duration. The agent is apparently hardy, surviving for approximately three years at the temperature of dry ice.[6, 7]

From the Division of Laboratories and Research, New York State Department of Health, Albany, and the Department of Medicine, Albany Medical College.

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METHODS

The volunteers used in the present study were nine men between 21 to 26 years of age in good health. They had not been exposed to epidemic gastroenteritis. They had remained asymptomatic during an outbreak of influenza A several weeks before the study commenced.

Techniques for isolation, preparation of fecal inoculum, and oral inoculation were those previously employed.[2] Inocula were fed in double gelatin capsules, each containing 0.5 milliliter.

The fecal inoculum was frozen at from −50° to −70° C. within ten minutes after collection on the first day of diarrhea from Volunteer No. 61 (T. H.) during the sixth human passage of the Marcy strain. It had been stored for eight months and was clarified by centrifugation. Cultures revealed no microorganisms and microscopic examination no ova or parasites.

This fecal suspension was used to initiate the tissue cultures. Liver and intestine were obtained from 3- to 5-month human embryos soon after death and from guinea pig embryos midway through development. Roller-tube and flask preparations were made with balanced salt solution[8] containing 20 per cent serum ultrafiltrate,[9] 6 per cent horse serum, and 6 per cent beef embryo extract. They were incubated at 35° C. for from one to three days before inoculation, and for from four to nine days after inoculation. The nutrient fluid was changed three or four times. Viability of tissues was proved by microscopy or explantation. Cultures were subinoculated with ground tissue suspended in nutrient fluid. From two to five serial passages were made.

RESULTS

The volunteers were inoculated first with tissue culture material, then re-inoculated with either fecal suspension or with sterile broth (Table I). They believed all to be infectious.

TABLE I. RESULTS OF INOCULATIONS

	FIRST INOCULATION			SECOND INOCULATION			
VOLUNTEER	INOCULUM	DOSE (ML.)	RESULT	INOCULUM	DOSE (ML.)	RESULT	INCUBATION PERIOD (HR.)
70 A. M.	Human emb. TC*	2.0	Possible† illness (60 hr.)	Sterile broth	5.0	No illness	
69 E. J.	Human emb. TC	2.0	No illness	Sterile broth	5.0	No illness	
71 S. McD.	Human emb. TC	2.0	No illness	Sterile broth	5.0	No illness	
63 J. S.	GP emb. TC‡	3.5	No illness	Fecal suspension	5.0	Severe illness§	58
64 C. W.	GP emb. TC	3.5	No illness	Fecal suspension	5.0	Moderate illness	59
65 D. P.	GP emb. TC	3.5	No illness	Fecal suspension	5.0	No illness	
66 J. M.	GP emb. TC	3.5	No illness	Fecal suspension	5.0	Possible illness	50
67 F. J.	Human and GP emb. TC‖	2.0	No illness	Fecal suspension	5.0	Mild illness	55
68 G. B.	Human and GP emb. TC	2.0	No illness	Fecal suspension	4.0	No illness	

*Human embryonic tissue culture.
†See Fig. 1 and case report.
‡Guinea-pig embryonic tissue-culture fluid.
§See Fig. 2 and case report.
‖Human and guinea-pig embryonic roller-tube tissue-culture fluid.

Volunteer No. 70 (A. M.) developed symptoms sixty hours after ingesting 2.0 ml. of second-passage human embryonic liver-intestine roller-tube tissue culture (Fig. 1). For this experiment two series of tubes were incubated for four and seven days after inoculation, respectively, in each passage. The tissue and fluid at the end of the second passage were pooled and fed to three subjects. The watery feces with which the tissue cultures were originally inoculated were diluted approximately 1,600,000 fold during the five fluid changes of the two passages, while the ground tissue was diluted approximately 400 fold.

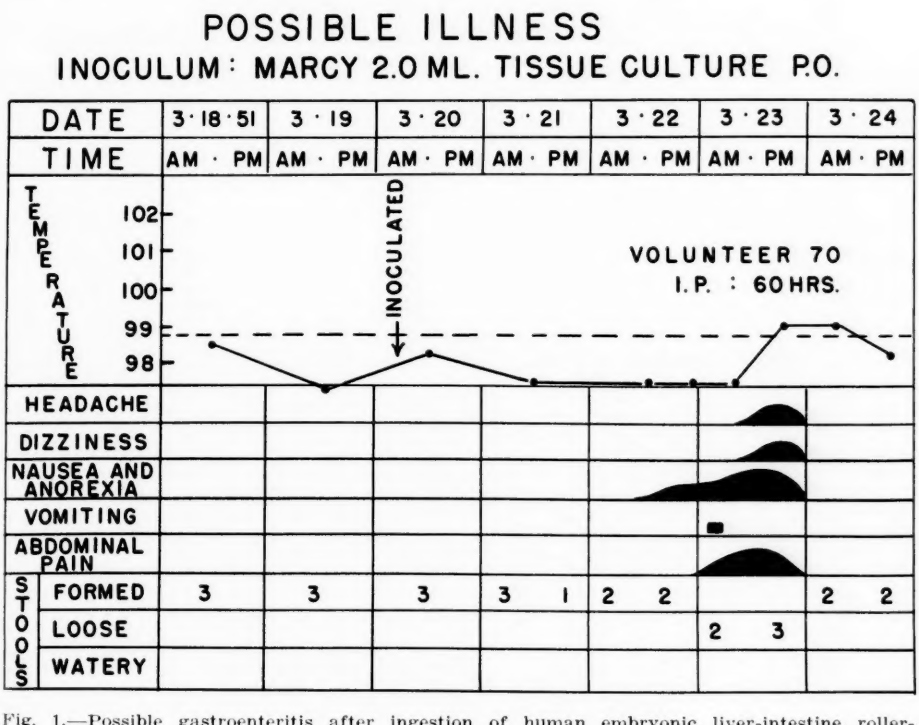

Fig. 1.—Possible gastroenteritis after ingestion of human embryonic liver-intestine roller-tube tissue culture material of the Marcy strain.

The patient reported slight anorexia and moderate nausea, malaise, dizziness, and abdominal discomfort, with one episode of vomiting and five loose (not watery) bowel movements in the first twenty-four hours of illness. Slight blood-streaking in the stools was attributed to a hemorrhoid. The patient was well the next day. While it was felt that this subject might have been somewhat unreliable in reporting his symptoms, except for diarrhea he was ignorant of what symptoms and signs to expect. He might have had a mild attack of the disease, since the incubation period and clinical syndrome were consonant with epidemic nonbacterial gastroenteritis.

There were no other illnesses following the feeding of tissue culture material. The second inoculation was done one hundred hours after the first. Three of six men fed stool suspensions had typical attacks[2]; a fourth possibly had the disease (Table I).

Volunteer No. 63 (J. S.) became ill with a characteristic severe attack of gastroenteritis fifty-eight hours after ingesting fecal suspension (Fig. 2). He awakened at 2 A.M. with urgent diarrhea. The feces were typically watery. The following morning he refused all food and had fleeting abdominal cramps. During the second day of illness he had

twenty watery stools, vomited five times, and suffered continuous severe nausea, abdominal cramps, headache, and dizziness. On the third day his symptoms remitted and feces were loose instead of watery. A day later headache, nausea, and vomiting again became severe, but he no longer suffered dizziness or abdominal pain. He became dehydrated and an infusion of 1,000 ml. of 5 per cent glucose in normal saline was given. The next day he was hungry and asymptomatic. It is noteworthy that even when the patient was dehydrated his temperature remained normal.

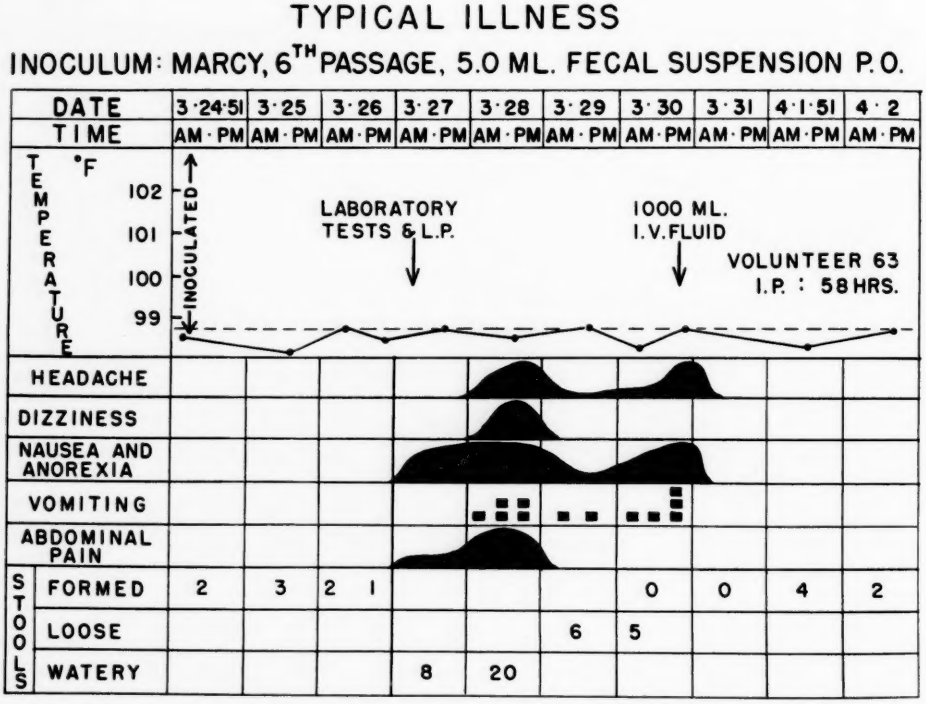

Fig. 2.—Characteristic severe gastroenteritis after ingestion of fecal suspension obtained from volunteer of the sixth human passage of the Marcy strain.

A series of clinical laboratory tests were done before the inoculations; at the time the men who received infectious fecal suspension became ill; and seven days after onset, approximately four days after subsidence of the attack in the most severely ill subject. The latter two series were limited to the six volunteers fed fecal suspension.

Complete examinations of the cerebrospinal fluid, blood, and urine revealed no abnormalities except for albuminuria and acetonuria in the dehydrated patients (Table II). Sedimentation rates, leukocyte and differential counts, and eosinophil counts were normal. The results of liver function tests indicated that hepatic dysfunction probably plays no part in the pathogenesis of epidemic nonbacterial gastroenteritis due to the Marcy strain (Table III). Serum amylase levels were within normal limits, and even serum electrolyte values of the acutely ill patients seemed little affected (Table IV).

TABLE II. RESULTS OF EXAMINATIONS OF CEREBROSPINAL FLUID, BLOOD, AND URINE

VOLUNTEER	RESULT OF INOCULATION	CEREBROSPINAL FLUID: CELL COUNT	PROTEIN (MG. %)	SUGAR (MG. %)	CHLORIDES (MEQ./LITER)	COLLOIDAL GOLD	BLOOD: SEDIMENTATION RATE CORRECTED (WINTROBE) (MM./HR.)	EOSINOPHIL COUNT PER CU. MM.	HEMOGLOBIN, P.C.V.*, W.B.C.† DIFFERENTIAL COUNT	URINE: ALBUMIN	ACETONE	pH, SPECIFIC GRAVITY, GLUCOSE, BILE, UROBILINOGEN, MICROSCOPIC
		I‡	I	I	I	I	C§ I	C I	C I 1 wk.‖	C I 1 wk.	C I 1 wk.	C I 1 wk.
63 J.S.¶	Illness	–**	61	65	130	B**	10 10	206 317	Normal	0 +†† 0	++++†† 0	Normal
64 C.W.	Illness	0	42	69	127	A/B‡‡	16 6	200 142	Normal	0 0 0	+++ ++	Normal
65 D.P.	No Illness	–	50	60	126	–	6 6	168 56	Normal	0 0 0	0 0	Normal
66 J.M.	? Illness	–	51	67	125	–	3 10	218 159	Normal	0 0 0	0 0	Normal
67 F.J.	Illness	0	33	60	117	A/B	9 13	193 113	Normal	0 0 0	0 0	Normal
68 G.B.	No Illness	–	33	58	118	–	6 12	68 167	Normal	0 0 0	0 0	Normal
Laboratory normal		0-2	15-30	45-80	120-130	A	0-15	150-300				

*Packed corpuscular volume.
†White blood count.
‡I = At time three subjects were acutely ill.
§C = Before inoculation.
‖1 wk. = One week later.
¶See Fig. 2 and case report.
**Some blood was admixed with the cerebrospinal fluid. This could account for the Type B colloidal gold curve.
††Patient had signs of dehydration.
‡‡Type A/B colloidal gold curves are of indeterminate significance.

TABLE III. RESULTS OF LIVER FUNCTION TESTS

VOLUNTEER	RESULT OF INOCULATION	ALKALINE PHOSPHATASE (mM UNITS)			BSP* 5 MG./KG. % RETAINED 45 MIN.			SERUM BILIRUBIN (MG. %) 1 MIN.			SERUM BILIRUBIN (MG. %) 30 MIN.			THYMOL TURBIDITY (MACLAGAN) UNITS			CEPHALIN-CHOLESTEROL FLOCCULATION 48 HR.			SERUM CHOLESTEROL MG. %		SERUM CHOLESTEROL % ESTERS		PROTHROMBIN TIME (QUICK) (%)		
		C†	I‡	1 wk.§	C	I	1 wk.	C	I	1 wk.	C	I	1 wk.	C	I	1 wk.	C	I	1 wk.	C	I	C	I	C	I	1 wk.
63 J. S.‖	Illness	1.2	1.2	1.4	4.2	15.4	6.4	0.09	0.06	0.06	0.55	0.60	0.22	4.8	3.3	3.1	0	0	++	182	197	68	73	40	50	50
64 C. W.	Illness	0.9	1.6	1.2	2.2	6.6	2.1	0.12	0.10	0.10	0.55	0.50	0.44	2.6	3.3	3.8	0	0	+++	160	191	68	73	40	34	34
65 D. P.	No illness	1.9	1.0	1.3	1.7	3.9	2.5	0.12	0.10	0.10	0.55	0.30	0.33	4.0	4.2	5.3	++	+	++	210	191	72	69	50	60	35.5
66 J. M.	?Illness	1.1	1.3	1.5	3.2	3.7	0.9	0.15	0.10	0.10	0.55	0.30	0.50	2.9	3.0	5.5	0	++	+++	160	120	77	74	32.5	50	45
67 F. J.	Illness	1.1	1.1	1.2	2.4	7.4	4.5	0.09	0.06	0.06	0.33	0.40	0.40	2.9	2.9	2.9	0	0	+	130	132	71	76	50	45	35.5
68 G. B.	No illness	1.4	1.7	1.6	2.4	6.6	5.0	0.12	0.10	0.10	0.49	0.30	0.44	4.2	4.6	6.2	+	++	++	222	240	73	72	55	55	45
Laboratory normal		0.9-2.8			<5			0-0.2			0.2-0.8			0-4.7			0-+++			140-320		69-74				

*Bromsulfalein retention test.
†C = before inoculation.
‡I = at time three subjects were acutely ill.
§1 wk. = one week later.
‖See Fig. 2 and case report.

TABLE IV. RESULTS OF SERUM AMYLASE, NONPROTEIN NITROGEN, AND ELECTROLYTE DETERMINATIONS

VOLUNTEER		INOCULATION RESULT OF	SERUM AMYLASE UNITS/100 ML.			NPN* (MG. %)		CO_2 MEQ./LITER		CHLORIDES MEQ./LITER		SODIUM MEQ./LITER		POTASSIUM MEQ./LITER	
			C†	I‡	1 wk.§	C	I	C	I	C	I	C	I	C	I
63	J. S.‖	Illness	85	80	36	31	35	22	18	103	118	141	137	4.2	4.8
64	C. W.	Illness	60	19	31	33	38	20	31	105	106	144	141	3.9	4.7
65	D. P.	No illness	58	34	22	31	36	23	23	104	107	142	142	3.9	4.4
66	J. M.	? Illness	64	36	52	34	36	26	23	104	108	144	143	4.9	4.5
67	F. J.	Illness	36	24	11	33	42	26	28	106	103	143	141	4.3	4.4
68	G. B.	No illness	60	46	50	31	37	24	32	102	102	142	139	4.1	5.1
Laboratory normal			10-160			25-40		25-34		100-108		139-147		3.8-4.7	

*Nonprotein nitrogen.
†C = Before inoculation.
‡I = At time three subjects were acutely ill.
§1 wk. = One week later.
‖See Fig. 2 and case report.

DISCUSSION

Since patients with epidemic nonbacterial gastroenteritis rarely need hospital care, biochemical and other clinical laboratory examinations are not obtained. An empirical series of observations was therefore made when an opportunity arose to obtain these data during etiologic studies in volunteers. The results shed no light on pathogenesis, and are unlikely to be of much aid in diagnosis except as it is useful to know that these tests may remain unchanged during the disease. Published reports suggest that changes in the central nervous system may be associated with this[10, 11] or similar[12] disease; but the fact that the cerebrospinal fluid was not altered in the present series confirms the single observation made previously[2] and shows that this examination is of little clinical value.

The outcome of the inoculations with human tissue culture material must be interpreted with caution. The group inoculated was small. The patient who reported symptoms had mild illness which might not have resulted from inoculation. The original stool suspension was diluted approximately 400 fold in the tissue during two culture passages. Kojima and associates[3] were successful in inducing gastroenteritis in one of two volunteers fed an 8,000 fold dilution of feces obtained from a Japanese outbreak in 1948 that resembled the Marcy outbreak in its clinical and epidemiologic features. Illness might have been due to survival of the agent in the tissue without propagation.

SUMMARY

A series of clinical laboratory examinations were performed on specimens from patients with experimental nonbacterial gastroenteritis induced by the Marcy strain. There were no striking changes in the values of the tests.

Results of inoculation of volunteers with tissue culture material are reported.

These experiments were done at the New York State Vocational Institution, West Coxsackie. The late John A. Lyons, Commissioner of the State Department of Correction, was unfailingly helpful, and our thanks are due also to William E. Leonard, Deputy Commissioner, for his whole-hearted support. We take pleasure in acknowledging our indebtedness and gratitude to Donald D. Scarborough, Superintendent, Joseph Conboy, Assistant Superintendent, and Dr. Solomon Yarvin, Senior Physician, of the Vocational Institution, and their respective staffs, for the generous cooperation which made this study possible. Leroy Weaver, Superintendent, and Dr. M. E. Pittman, Senior Physician, of the Elmira Reformatory, Elmira, New York, also gave us helpful aid and support. We wish to express our sincere appreciation to Dr. Donald G. Remp of the Department of Biochemistry, Albany Medical College, for performing the liver function and other chemical tests.

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PREVENTION OF PARALYTIC POLIOMYELITIS IN TONSILLECTOMIZED CYNOMOLGUS MONKEYS BY HUMAN GAMMA GLOBULIN

J. M. Adams, M.D., R. A. Boak, M.D., C. M. Carpenter, M.D., J. D. French, M.D., S. J. Klein, Ph.D., J. J. Pressman, M.D., and J. L. Smith, M.D.
Los Angeles, Calif.

AN INCREASED susceptibility to poliomyelitis has been demonstrated in tonsillectomized cynomolgus monkeys fed virus for three to five days after operation.[1, 2] By means of the administration of concentrated human gamma globulin, it has been possible to protect such tonsillectomized monkeys from the paralytic forms of poliomyelitis. It has likewise been found that when monkeys are given gamma globulin to prevent or modify the paralytic forms of the disease they will still develop neutralizing substances in their blood against the strain of virus which has been fed.[2]

Recently Bodian[3] and Horstmann[4] detected the virus of poliomyelitis in the blood of monkeys following the feeding of virus, and, additionally, showed that gamma globulin protected the animals. Our studies in mice and in monkeys have failed to show protection when large doses of gamma globulin were given *during* the feeding of virus to monkeys and *following* intracerebral inoculation into mice. However, if sufficiently large doses are given *prior* to exposure, good protection has been demonstrated.

In four different experiments, 156 animals fed poliomyelitis virus have been studied; of this total, 117 were tonsillectomized and 39 animals not operated made up the control groups. Our primary purpose was to demonstrate the protective effect against the disease of concentrated human gamma globulin in tonsillectomized monkeys and to determine the dose necessary for modification or prevention. It has been known for some time that human gamma globulin contained immune substances for all three types of poliomyelitis.[5] These original studies were performed by the administration of previously mixed virus and gamma globulin and therefore represented test-tube or in vitro experiments. Several thousand times more gamma globulin is required to protect animals against poliomyelitis when inoculated intramuscularly and probably represents more nearly the true protective dose. It is important to remember that human gamma globulin when given to monkeys is a heterologous substance, and probably a larger dose is required than if homologous glob-

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From the Investigation Medicine Service, Veterans Administration Hospital, Long Beach, California, and the Departments of Pediatrics, Infectious Diseases and Surgery, School of Medicine, University of California at Los Angeles.

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ulin were used. The dose, therefore, needed to protect human beings with human gamma globulin is undoubtedly somewhat smaller than that required to protect monkeys. The validity of this concept can be determined satisfactorily only by studies in human beings.

The possible modifying effect on induced poliomyelitis by simultaneous infections with distemper and Coxsackie viruses was studied. It has been suggested by reports in the literature that some interference or inhibition of one virus disease might occur when another virus infection is present in the same host.[6, 7] It, therefore, seemed plausible to attempt to study this phenomenon under these experimental conditions. Potassium chloride was added to the diet of one of the tonsillectomized groups as a trial experiment, since a relationship between hypopotassemia and the paralytic stages of familial periodic paralysis exists, and it has been reported to have a possible relationship to the paralytic forms of poliomyelitis.

MATERIALS AND METHODS

The procedures followed in the present study were, in general, similar to those described in a previous report.[2] The principal change concerned the time of administration of the gamma globulin with reference to the feeding of virus and the dosage employed. The animals in each group were similarly anaesthetized with Nembutal intraperitoneally. The virus of poliomyelitis which we employed was the Type 2, Y-SK strain.* This virus was adapted to the cynomolgus monkey by passage and was fed on bread to all of the animals beginning on the day of operation. Ninety cynomolgus monkeys weighing from 3 to 5 pounds were employed in two experiments, designated at III and IV, inasmuch as they are based upon, but supplement previously published data involving Experiments I and II. In Experiment III, the dose was 0.5 ml. of a 10 per cent monkey cord suspension given daily for five days; in Experiment IV, a dose of 2.0 ml. of a 2 per cent suspension was fed for three days.

In Experiment III, 38 monkeys were tonsillectomized and 8 animals were not operated. The animals were divided into five groups as follows:

Group I—10 monkeys, tonsillectomized.

Group II—9 monkeys, tonsillectomized and injected with human gamma globulin. One-third of the total dose of 15 ml. per animal was administered intramuscularly following the operation and prior to the feeding of virus, and on the third and fifth days. The average weight of the animals in this group was 3.85 pounds, and the total dose per animal was approximately 4 ml. per pound.

Group III—10 monkeys, tonsillectomized and injected subcutaneously with 1.0 ml. distemper virus (40 per cent chorioallantoic membrane suspension of egg-adapted distemper virus), following the operation and prior to the feeding of poliomyelitis virus.

Group IV—9 monkeys, tonsillectomized and fed potassium chloride, 2 gm. per animal, daily for the duration of the experiment.

Group V—8 control monkeys, nontonsillectomized and fed poliomyelitis virus.

In Experiment IV, 44 monkeys challenged with poliomyelitis virus were divided into four equal groups as follows:

*Kindly provided by J. R. Paul, M.D.

Group I—11 monkeys, tonsillectomized.

Group II—11 monkeys, tonsillectomized. 10 were injected intraperitoneally with 1.0 ml. tissue suspension* of Coxsackie B virus—Conn. 5, and one with Coxsackie A virus—California 1.

Group III—11 monkeys, tonsillectomized and injected with concentrated human gamma globulin. 2 ml. per pound were inoculated intramuscularly in a single dose the day before the operation.

Group IV—11 monkeys, nontonsillectomized.

The animals which died were autopsied and others showing evidence of illness were sacrificed, either immediately after symptoms developed, or at the end of the experiment. The criterion for poliomyelitis infection in the animals was the demonstration of characteristic pathologic lesions in the cord.

White mice, in groups of 8, were used in neutralization tests. The virus suspension was titrated in mice and the LD_{50} was determined to be $10^{-3.4}$. Each mouse received 50 LD_{50} in the neutralization tests.

RESULTS

In Experiment III, during the first five days an epizootic developed among the animals which resulted in 14 deaths. These animals were eliminated from the results of this experiment as necropsy studies revealed no signs of poliomyelitis and they all expired within the incubation period of experimental poliomyelitis.

Thirty-two animals survived the epizootic and form the basis for the results of this experiment.

In Group I, tonsillectomized only, 5 of 6 animals died, and their spinal cords showed typical lesions of poliomyelitis.

In Group II, tonsillectomy plus gamma globulin, one animal of seven died and showed signs of poliomyelitis in the spinal cord. The monkey was the largest in the group, weighing 5 pounds, and received a total of 3 ml. per pound divided into three doses over a period of five days. The convalescent blood samples of 4 of the surviving animals, when tested in mice, revealed the results tabulated in Table I.

TABLE I. RESULTS OF NEUTRALIZATION TESTS ON SERUM FROM MONKEYS

SOURCE OF SERUM MONKEY NO.	RESULTS	
	21 DAYS	30 DAYS
212	6/8	6/8
215	0/8	5/8
217	3/8	3/8
219	0/8	1/8
Control	5/8	7/8

The numerator indicates the number of animals which died: the denominator, the number of animals tested.

In Group III, tonsillectomy plus distemper virus, 6 of 7 animals died with proved poliomyelitis by histologic examination of the spinal cords. The con-

*The inoculum was a 10 per cent suspension of infected suckling mouse tissue in 0.85 per cent salt solution.

valescent blood sample on the one surviving animal in the group showed no evidence of neutralizing substance.

In Group IV, tonsillectomy plus potassium chloride, 5 of 5 animals died of poliomyelitis.

In Group V, nonoperated animals, 1 of 7 animals died of poliomyelitis. The convalescent serum test on 6 of these animals is shown in Table II. Monkey serum number 230 revealed evidence of solid protection for mice against poliomyelitis.

TABLE II. RESULTS OF NEUTRALIZATION TESTS ON SERUM FROM MONKEYS

SOURCE OF SERUM MONKEY NO.	RESULTS	
	21 DAYS	30 DAYS
230	0/8	0/8
232	5/8	6/8
233	6/8	6/8
234	5/8	5/8
235	7/8	8/8
237*	3/8	4/8
Control	5/8	7/8

The numerator indicates the number of animals which died; the denominator, the number of animals tested.

*The cord sections on this monkey showed positive evidence of poliomyelitis.

A résumé of the data in this experiment indicates that some protection was afforded the animals by the administration of concentrated human gamma globulin when given in doses of approximately 4 ml. per pound over a five-day period. The only animal which developed poliomyelitis received a total of 3 ml. per pound. The tonsillectomized animals had a much higher attack rate than the nonoperated controls. Distemper virus and the feeding of potassium chloride had no measurable effect on the susceptibility to infection.

In experiment IV, 33 cynomolgus monkeys were tonsillectomized on May 13, 1952. Eleven animals were not operated but all were anaesthetized with Nembutal. The results in four groups are as follows:

In Group I, tonsillectomy only, 9 of 11 animals died of poliomyelitis as shown by histologic examination of the spinal cord.

TABLE III. RESULTS OF NEUTRALIZATION TESTS ON SERUM FROM MONKEYS

SOURCE OF SERUM MONKEY NO.	RESULTS	
	21 DAYS	30 DAYS
2	0/8	0/8
3	3/8	4/8
4	4/8	5/8
5	1/8	3/8
6	8/8	8/8
7	3/8	5/8
8	7/8	7/8
9	3/8	4/8
10	3/8	5/8
11	8/8	8/8
Control	8/8	8/8

The numerator indicates the number of animals which died; the denominator, the number of animals tested.

In Group II, tonsillectomy plus Coxsackie B and A, all of the animals died, and 10 of the 11 had positive spinal cord evidence of poliomyelitis. Forty-eight hours after inoculation with Coxsackie viruses, these animals all had rhinorrhea, consisting of a rather watery, whitish discharge. Pharyngeal washings and stool specimens were examined for Coxsackie viruses and the same strains were recovered. No sparing effect was evident, but, to the contrary, 3 of the monkeys died earlier than any of the animals in the other groups and 2 of these had positive cord findings, identical with those of poliomyelitis. There was no clinical evidence of paralysis in these animals, but generalized weakness was evident the day before death.

In Group III, tonsillectomy plus gamma globulin, all 11 animals survived with no signs of poliomyelitis or paralysis. One animal which showed some tremor was sacrificed on June 6, and the spinal cord was normal. Convalescent blood samples on 10 of these animals were tested in mice for evidence of neutralizing substances. The results are reviewed in Table III.

The remaining 10 surviving animals of this same group (Group III) were rechallenged on July 1, 1952, by oral administration for three days, with an increased dose of the same virus previously employed. On July 14, Monkey No. 4 was found dead in his cage, and histologic study of the spinal cord revealed clear-cut evidence of poliomyelitis. The remaining 9 animals have continued well, with no signs of illness or paralysis.

In Group IV, no operation, 4 of 11 monkeys became paralyzed and either died or were sacrificed on June 6. The spinal cords of these animals were all positive.

A résumé of the data in Experiment IV indicates complete protection of all 11 monkeys given human gamma globulin in a dose of 2 ml. per pound, administered in a single injection, eighteen hours before operation and twenty-four hours prior to the start of virus feedings. The Coxsackie viruses as given, although producing purulent rhinitis, had no effect on the incidence or severity of the poliomyelitis, and again the tonsillectomized animals had a much higher attack rate than did the nonoperated controls.

DISCUSSION

Although we did not set out to study the relationship of tonsillectomy to poliomyelitis, it became apparent from the accumulating data that the attack rate in these animals was considerably increased over that observed in the nonoperated animals. When we eliminate the tonsillectomized animals which were given gamma globulin from all of the experiments, we have 41 of 63 animals (including those previously reported[2]) with proved poliomyelitis among the tonsillectomized monkeys. This incidence represents an attack rate of 65 per cent, and is in agreement with the studies of Von Magnus and Melnick.[1] Furthermore, Faber and associates[8] demonstrated 100 per cent attack rate of bulbar poliomyelitis in monkeys, given virus prior to tonsillectomy, using fairly large doses of Type I virus. The experimental evidence that tonsillectomy increases the incidence of poliomyelitis in monkeys, therefore, no longer needs to be a controversial subject.

In our initial report[2] it was apparent that the data failed to show evidence of protection of monkeys infected experimentally by oral feedings of poliomyelitis virus, when up to 2 ml. of concentrated human gamma globulin were given in divided doses per pound of animal weight, after the animal had been challenged. Even in these experiments, however, the attack rate of operated animals which received gamma globulin after exposure to the virus was not appreciably higher than in those of the control nonoperated groups, and certainly far below that found to exist in the total group of operated, unprotected animals. In all monkeys which received gamma globulin in varying doses prior to or after tonsillectomy, the attack rate was 5 of 41, or 12 per cent. This is approximately identical with the results in nonoperated groups of which 5 of 38 animals, or 13 per cent, became infected with poliomyelitis; indicating that the protection afforded approximated the natural resistance to infection of a sample of normal monkey population. When gamma globulin is administered *before* challenge, however, infectivity rate would seem to approach zero. Thus, it appears to be clearly indicated that protection is afforded when 2 ml. per pound of body weight is administered to the animal prior to the virus feeding. These findings have been confirmed by mouse studies, to be reported elsewhere, in which gamma globulin administered four days before the intracerebral inoculation of virus afforded a maximum protection. Adequate as this protection appears to be for both mouse and monkey, it must be remembered that human serum (gamma globulin) was employed. It would seem extremely likely that less human gamma globulin would be necessary to protect man.

Some evidence is presented from these studies which might indicate the development of active immunity in the monkey receiving gamma globulin and displaying no evidence of clinical poliomyelitis after challenge with the virus. Nine of ten surviving animals in this group successfully resisted rechallenge. Furthermore, mouse inoculation studies strongly suggested that the serum of surviving asymptomatic animals contained protective antibodies. Thus, in the serum of 3 of 4 such animals, antibodies were found to exist which significantly protected mice from intracerebral inoculation (Experiment III, Group II). The same tendency was found to exist in surviving animals from Group III in the last experiment; 6 of 10 sera showing some mouse protection.

A recent review of the literature on the tonsillectomy-poliomyelitis problem by Mills[9] indicates unmistakable evidence that the attack rate of bulbar poliomyelitis in many epidemics has been found to be significantly higher on recently tonsillectomized patients, and evidene is increasing that bulbar poliomyelitis, rather than the spinal form of the disease, is more likely to occur in patients tonsillectomized at any time than in those with tonsils intact. He concludes that "Adeno-tonsillectomy should not be performed during an epidemic."

Conversely, Miller[10] has expressed the opinion that "Operations on the nose and throat, including tonsillectomy and adenoidectomy, either recent or done long before, had no significant effect, statistically, on the incidence of

poliomyelitis in Los Angeles County during the three-year period, 1949-1951." He admits, however, that the summer months are those in which the fewest tonsillectomies are done, presumably because of the general impression among physicians that infection is higher after operation during the epidemic season. For precisely this reason, it would be essential to know the actual number of tonsillectomies made during the summer period in comparison to the post-operative incidence of poliomyelitis in order to accept Miller's conclusion. Thus, in the year 1951, he reported 10 patients with poliomyelitis following operation (tonsillectomy and adenoidectomy) of which 8 occurred during the July to October period. Had there been only 16 operations during this time, for example, the incidence of associated infection wuold have been alarmingly high.

Our experimental data at this time do not permit extrapolation to the human tonsillectomy-poliomyelitis problem; yet the incidence of infection after recent operation in animals has been so consistently high, that acknowledgement of a relationship between the two events is unavoidable. Our experimental evidence indicates that a high degree of protection can be conveyed to the animal if the gamma globulin is administered before operation. Implicit in this observation is the suggestion that maximum protection might be afforded man by administration of gamma globulin at the times of exposure to infection, or to a stressful situation (such as operation) which might make him more susceptible to infection in epidemic periods. The validity of these considerations must await the accumulation of more human data similar to that described above.

SUMMARY

Tonsillectomized cynomolgus monkeys were protected from paralytic poliomyelitis by human gamma globulin when a dose of 2 ml. per pound was administered, *prior* to feeding virus. Nine of ten passively protected animals have resisted rechallenge with the poliomyelitis virus, suggesting that the monkeys acquired immunity.

The poliomyelitis attack rate in the tonsillectomized animals was 65 per cent; in the animals which were tonsillectomized and given gamma globulin, it was 12 per cent; and in the nonoperated control animals, it was 13 per cent. In Experiment IV, 9 of 11 tonsillectomized monkeys and 10 of 11 tonsillectomized monkeys also given Coxsackie virus, developed poliomyelitis. None of the 11 animals injected with human gamma globulin prior to operation, developed paralytic disease.

An attempt to inhibit poliomyelitis in monkeys, by means of simultaneous infections with distemper or Coxsackie viruses, failed. The animals given Coxsackie viruses intraperitoneally developed profuse nasal discharge forty-eight hours after inoculation, and the viruses were isolated from this material as well as from the stools.

Tonsillectomy markedly increased the attack rate of paralytic poliomyelitis.

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RECENT CHANGES IN SENSITIVITY OF MICROCOCCUS PYOGENES TO VARIOUS ANTIBIOTIC AGENTS

GERALD M. NEEDHAM, PH.D.,* AND DONALD R. NICHOLS, M.D.**
ROCHESTER, MINN.

INFECTIONS caused by *Micrococcus pyogenes* var. *aureus (Staphylococcus aureus)* continue to be a difficult problem because at present many of the strains of micrococci encountered clinically are resistant not only to penicillin but also to one or more of the other antibiotics. In 1949, we reported observations on the large number of infections caused by strains of micrococci that were resistant to penicillin and to streptomycin.[1] At that time all the strains studied were sensitive to aureomycin. Since 1949, several investigators[2-6] have reported an increasing incidence of strains of penicillin-resistant micrococci that are resistant to other antibiotics as well.

Our present report deals with observations made at varying intervals since 1948 in an attempt to determine whether any change had occurred in the resistance of micrococci to various antibiotics, including penicillin, streptomycin, aureomycin, terramycin, chloramphenicol, and erythromycin. Numerous investigators, including Rountree and associates[5] and Barber and co-workers,[7] have noted the occurrence of nasal carriers of resistant micrococci in hospital personnel and healthy adults. Therefore, a study also was made of the incidence of nasal carriers of micrococci in nurses in three of the Rochester hospitals and in a number of outpatients seen in one of the general medical sections of the Mayo Clinic.

MATERIAL AND METHODS

During each of five periods of investigation an attempt was made to isolate *Micrococcus pyogenes* from all clinical material submitted to the Laboratory of Clinical Bacteriology of the clinic. When this organism was isolated, determinations of its sensitivity to various antibiotics were made by one of two methods. For the most part the tests were carried out on freshly prepared blood-agar plates in which the respective antibiotics were incorporated. During the periods January, 1951, through March, 1951, and October, 1951, through November, 1951, absorbent paper disks impregnated with the antibiotic being tested were also used. Three commercially available preparations and some prepared in our own laboratory were used.

Uniform amounts of a pure culture that had been incubated for six hours were spread evenly over the surfaces of a series of blood-agar plates that contained twofold dilutions of the antibiotic. The plates were incubated at 37° C. for twenty-four hours, and the sensitivity was recorded as that concentration of antibiotic which completely inhibited growth of the organisms. A standard strain of *Micrococcus pyogenes* that had a known degree of sensitivity was used as a control. When the paper disks were used, the inoculum was spread evenly over the surface of the blood-agar plate and allowed to dry for approximately one hour before the disks were put in place. The plates were incubated in an inverted position at 37° C. for twenty-four hours. The zone of inhibition was measured and recorded. At least two disks that contained different concentrations of each antibiotic were used. If any

From the Section of Bacteriology* and the Division of Medicine,** Mayo Clinic.
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irregularity in the size of the zone of inhibition induced by the disks was exhibited, the strain was tested by the first-described method.

RESULTS

Observations on Clinical Material From Hospitals.—For purposes of comparison, all data obtained from studies of sensitivity conducted on clinical material have been included in Fig. 1. The material for the first three periods was selected to the extent that studies for sensitivity to streptomycin and aureomycin were made only on penicillin-resistant micrococci. For the remaining two periods of study, sensitivities for each of the antibiotics were determined for all strains. In only three instances was resistance for any of the other antibiotics demonstrated in a penicillin-sensitive strain.

During the period from December, 1948, through January, 1949, 50 strains of *Micrococcus pyogenes* were isolated from various clinical materials. Growth of 34 (68 per cent) of these was not inhibited by 1.6 Oxford units of penicillin per cubic centimeter of culture medium. In addition, growth of 14 of these 34 penicillin-resistant strains was not inhibited by 25 micrograms of streptomycin per cubic centimeter of culture medium. Growth of all 34 penicillin-resistant strains was found to be inhibited by as little as 0.78 μg of aureomycin per cubic centimeter of culture medium.*

During the period from March, 1949, through June, 1949, another 50 strains of *Micrococcus pyogenes* were isolated from various clinical materials. Growth of 25 of these was not inhibited by 3.2 units of penicillin. To be certain that it was not necessary to test the organism to more than 3.2 units of penicillin, 34 strains of *Micrococcus pyogenes* whose growth was not inhibited by this concentration were subjected to greater concentrations of penicillin. All but 8 of these grew equally well in the presence of 50 units of penicillin. The remaining 8 strains grew well in 25 units but were unable to grow in 50 units. It was presumed, therefore, that for practical purposes strains capable of growth in the presence of 3.2 units of penicillin could be considered resistant to penicillin. Growth of 7 of the 25 penicillin-resistant strains was not inhibited by 25 μg of streptomycin. The growth of all 34 strains was inhibited by 0.78 μg of aureomycin or less.

During the period from November, 1949, through February, 1950, 100 strains of *Micrococcus pyogenes* were isolated. Growth of 52 of these was not inhibited by 3.2 units of penicillin; growth of 14 of these 52 strains was not inhibited by 25 μg of streptomycin. Growth of all 52 strains was inhibited by 6.2 μg or less of aureomycin or terramycin.

From January, 1951, through March, 1951, 100 strains of *Micrococcus pyogenes* were isolated. Growth of 42 of these was not inhibited by 3.2 units of penicillin; growth of 8 of the 100 strains was not inhibited by 25 μg of streptomycin. Growth of 9 of the 100 strains was not inhibited by 6.2 μg of aureomycin; these 9 strains were also resistant to 6.2 μg of terramycin.

*To avoid repetition, the expression "per cubic centimeter of culture medium" will be omitted in succeeding instances wherein concentration of an antibiotic is given.

One hundred strains of *Micrococcus pyogenes* were isolated in October and November, 1951. Growth of 64 of these was not inhibited by 3.2 units of penicillin; growth of 40 of the 100 strains was not inhibited by 25 μg of streptomycin. Thirty-six strains were resistant to 6.2 μg of aureomycin or terramycin. One of these strains was resistant to 25 μg of chloramphenicol (Chloromycetin).

During the spring of 1952, 86 strains of *Micrococcus pyogenes* were tested for their sensitivity to erythromycin (ilotycin).* Two of the strains, which were sensitive initially, increased in resistance during treatment and subsequently were not inhibited by 50 μg of erythromycin. These strains were considered to have developed resistance as a result of treatment, since no naturally

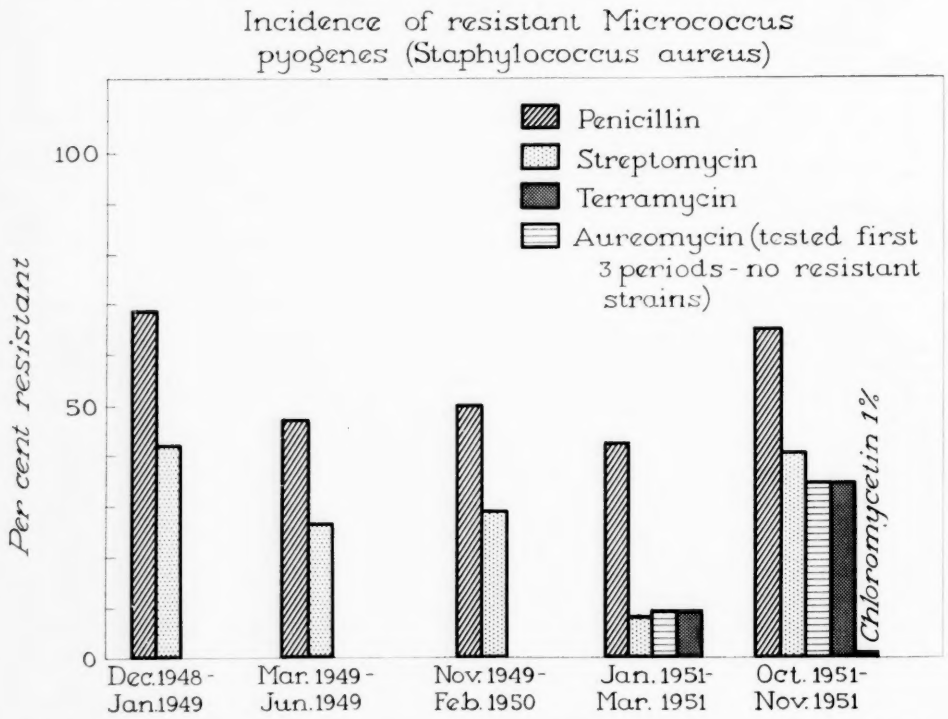

Fig. 1.—Incidence of resistant *Micrococcus pyogenes* (*Staphylococcus aureus*) isolated during five intervals from material submitted for bacteriologic study.

occurring resistance could be demonstrated in 34 strains that had been isolated the previous year and maintained in a frozen state. One of 75 strains of coagulase-negative micrococci was found to be naturally resistant to erythromycin. Preliminary studies revealed that none of these 3 resistant strains produced a substance acting against erythromycin. Most of the sensitive strains were inhibited by as little as 0.8 μg of erythromycin.

A study was made of the records of 31 patients from whom strains of *Micrococcus pyogenes* resistant to penicillin, aureomycin, and terramycin were isolated in 1951. Postoperative wounds were the most frequent source (16

*The erythromycin used in this study was kindly supplied by the Department of Research, Eli Lilly & Company, Indianapolis, Ind.

patients). In 2 patients the organisms were obtained from purulent cutaneous infections; both these patients had diabetes mellitus. Seven of 8 patients who had resistant micrococci in the urine had experienced previous urologic operations or catheterization. The other had carcinoma of the prostate. Two of 3 patients who had bacteremia had undergone recent abdominal operations and one of these died from acute bacterial endocarditis. The third patient had extensive pemphigus. Resistant micrococci were recovered from both cerebrospinal fluid and a chronically infected mastoid in one patient. In one patient the resistant organism was obtained from the ankle.

Observations on Outpatients.—During the summer and early fall of 1951, 100 outpatients who came for general examination in a medical section of the clinic and who had no evidence of infection of the upper part of the respiratory tract were examined as possible carriers of *Micrococcus pyogenes*. Cultures were prepared from a swab that was inserted into the anterior nares and smeared immediately on blood agar. Organisms grown from colonies picked from the plates were tested for their ability to coagulate plasma. Coagulase-positive strains were studied for their susceptibility to antibiotic agents. Twenty-five patients had a sufficient number of micrococci to be classified as carriers. The growth of 10 of these strains was not inhibited by 3.2 units of penicillin but all strains were sensitive to aureomycin, terramycin, streptomycin, and chloramphenicol.

Observations on Hospital Nurses.—During the same period in which the outpatients were studied, 100 nurses who worked primarily with hospitalized medical patients were examined in the same manner for the presence of *Micrococcus pyogenes*. Forty of them had a sufficient number of micrococci to be classified as carriers. The growth of 20 strains was not inhibited by 3.2 units of penicillin. The growth of 3 strains was not inhibited by 6.2 μg of aureomycin or terramycin. The growth of 6 strains was not inhibited by 25 μg of streptomycin, and the growth of one strain was not inhibited by 25 μg of chloramphenicol.

One nurse carried a strain that was resistant to streptomycin but sensitive to the other antibiotics. Two nurses carried strains resistant to both penicillin and streptomycin, and 2 nurses carried strains resistant to penicillin, streptomycin, aureomycin, and terramycin. Another carried a strain resistant to all these four antibiotics, and also to chloramphenicol.

COMMENT

Prior to 1949, the incidence of penicillin-resistant strains of micrococci isolated from clinical material submitted to this laboratory appeared to increase. However, since the beginning of 1949, the incidence of penicillin-resistant strains has varied only slightly, and no significant increase has occurred. Of interest is the fact that the ratio of penicillin-resistant strains to penicillin-sensitive strains in our studies is comparable to that reported in recent years by investigators at several other institutions.[4, 5] It would appear at the present time at least that a rough equilibrium between penicillin-sensitive and penicillin-

resistant strains may have been reached in patients hospitalized in Rochester. A reservoir of penicillin-resistant micrococci appears to exist in the form of carriers of these organisms among medical and nonmedical personnel in hospitals. Twenty per cent of 100 healthy nurses carried penicillin-resistant micrococci. However, only 10 per cent of 100 outpatients carried penicillin-resistant micrococci at the same time. Thus, no significant increase has occurred apparently in the incidence of penicillin-resistant micrococci among outpatients coming under our observation.

Resistance of *Micrococcus pyogenes* to streptomycin has varied widely. The incidence of streptomycin-resistant strains among the micrococci isolated in this laboratory decreased rapidly after administration of this drug by itself was discarded largely in favor of other antibiotics. However, since the increased use of mixtures of penicillin and streptomycin in hospitals during 1951, the incidence of streptomycin-resistant strains appears again to have increased.

In 1949, all the penicillin-resistant strains of micrococci were found to be sensitive to as little as 0.78 μg of aureomycin. In the early part of 1951, 9 per cent of all strains of *Micrococcus pyogenes* were resistant to more than 6.2 μg of aureomycin and, in the late part of 1951, 36 per cent have been found to be resistant to aureomycin. Most of these resistant strains were obtained from hospitals where either aureomycin or terramycin was used in the preoperative preparation of patients. The fact that no strains resistant to aureomycin or terramycin were found in the 100 outpatients in 1951 is of interest. The 3 healthy nurses who had organisms resistant to aureomycin and terramycin never had received either aureomycin or terramycin.

All the strains resistant to aureomycin were resistant also to terramycin. This observation is consistent with previous reports on the pattern of resistance induced in vitro to both aureomycin and terramycin when the organism is exposed to increasing concentrations of either antibiotic alone. Terramycin has been used in increasing amounts in the hospitals in Rochester both in the treatment of infections and in the preoperative preparation of patients for various surgical procedures. The increasing incidence in these hospitals of micrococci resistant to terramycin is, therefore, to be expected.

Only 2 strains that were not inhibited by 25 μg of chloramphenicol have been encountered in our studies. This is probably related to the fact that few patients have received this antibiotic in the hospitals in Rochester. Therefore, the appearance of any significant number of chloramphenicol-resistant strains in these hospitals would not be expected. Because of the potential toxicity of chloramphenicol, particularly in the development of aplastic anemia, other antibiotics are used whenever possible. However, in patients who are seriously ill from infections caused by strains of micrococci that are resistant to other antibiotics, we feel justified in using chloramphenicol in spite of the risk.

Because many investigators have found that neomycin and bacitracin frequently cause serious toxic reactions when administered systemically, we have used these antibiotics systemically only in rare instances.

Observations concerning erythromycin, one of the newest of the antibiotic agents, are of considerable interest. As previously mentioned, we have studied 34 strains of *Micrococcus pyogenes* that were collected prior to clinical use of

erythromycin and 86 strains that were collected during the early period of investigation of this antibiotic. None of the strains were naturally resistant to erythromycin, even though they showed pronounced resistance to the other antibiotic agents previously studied. Heilman and associates[8] reported good results from use of erythromycin in several patients who had infections owing to strains of *Micrococcus pyogenes* resistant to other antibiotics. However, these investigators established the fact that *Micrococcus pyogenes* possesses the ability to develop resistance to erythromycin. They found that different strains vary as to the rapidity with which they develop this resistance. In one of their patients and in one observed since their report, the development of resistance has been observed clinically. In both instances, the organisms were sensitive to erythromycin when originally tested but became resistant to more than 50 μg during or soon after prolonged treatment with the drug. Both patients had chronic osteomyelitis, and the organism was cultured directly from the bone. Therefore, erythromycin appears to be of value, but the ease with which certain strains of *Micrococcus pyogenes* become resistant to it apparently will limit its usefulness in infections caused by this organism.

Analysis of the records of the 31 patients from whom strains of *Micrococcus pyogenes* resistant to penicillin, aureomycin, and terramycin were isolated revealed that these resistant strains usually were associated with infections that developed while the patients were hospitalized. In most instances these resistant organisms were recovered from postoperative wounds or from urine after urologic operations or catheterization. These findings are in accord with those which others have reported.[2, 9] However, in our group of cases, these resistant organisms also were isolated occasionally from patients who had deep closed infections and who had not been hospitalized previously. The pyogenic infection of the ankle is an example of such. The patient gave no history of an injury or previous infection. He had not been hospitalized previously nor had he received any antibiotic therapy.

These deep infections caused by resistant organisms are few, however, and the problem of infection from strains that are resistant to multiple antibiotics appears at present to be important primarily as regards cross infection among hospitalized patients. It has been mentioned that medical and nonmedical personnel may serve as a reservoir for resistant organisms. While infections caused by micrococci that are resistant to multiple antibiotics are often mild, these organisms may cause serious and occasionally fatal infections. One of our patients died from acute bacterial endocarditis caused by a resistant strain; death occurred in another from fulminating meningitis. The importance of the prevention of cross infections with these organisms and of the control of carriers is obvious. Indiscriminate use of antibiotic agents intensifies the problem and is to be condemned strongly.

SUMMARY

During each of five periods since 1948, all strains of *Micrococcus pyogenes (Staphylococcus aureus)* isolated at the Mayo Clinic from clinical materials have been tested for sensitivity to various antibiotic agents.

The number of penicillin-resistant strains of *Micrococcus pyogenes* among hospitalized patients had reached 60 per cent in 1948; this number has not increased since that time. The incidence of streptomycin-resistant strains was 41 per cent in 1948; it decreased during the years that the use of streptomycin was omitted largely in favor of other antibiotics, but the incidence has increased since mixtures of penicillin and streptomycin have been in frequent use.

No strain of micrococci that was resistant to aureomycin was found in 1948. Subsequently, the incidence of strains resistant to aureomycin and terramycin has gradually increased, reaching a value of 36 per cent in November, 1951. The incidence of strains resistant to chloramphenicol over these years has increased only slightly, presumably because chloramphenicol is infrequently used in these hospitals.

No strain of *Micrococcus pyogenes* naturally resistant to erythromycin has been encountered, but in 2 patients a strain that originally was sensitive to erythromycin became resistant during or soon after treatment with this antibiotic.

When strains of *Micrococcus pyogenes* were encountered that were resistant to penicillin, aureomycin, and terramycin, they were obtained usually from postoperative wounds or from urine after urologic operations or catheterization. Occasionally, however, resistant strains have been isolated from patients who have deep closed infections and in whom previous antibiotic therapy has not been given. Infections caused by strains of micrococci resistant to multiple antibiotics usually were mild, but several fatal infections did occur.

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LABORATORY METHODS

QUANTITATIVE DETERMINATION OF NITRATE BY ENZYMIC REDUCTION TO NITRITE

WILLIAM R. STRAUGHN, M.S., INEZ GREENE TRAVIS, M.S., AND
EDWIN P. HIATT, PH.D., M.D.
CHAPEL HILL, N. C.

MOST of the available methods for the quantitative determination of nitrates are either insufficiently sensitive or require rather time-consuming procedures.

The method described is relatively simple to perform and is quantitatively sensitive for the determination of 0.01 meq per liter of nitrate. The principle involved is that of quantitative reduction of nitrate to nitrite by bacterial nitratase followed by a colorimetric determination of the nitrite from which, by means of a standard curve, the nitrate concentration is calculated. The use of this principle was reported by Wainwright[1] in his studies on nitratase production.

In this paper, a procedure is described for the determination of nitrate using a cell suspension of *Escherichia coli* under aerobic conditions.

METHODS AND MATERIALS

A. *Preparation of the Cell Suspension.*—The organism is *Escherichia coli*, Crookes strain, American Type Culture Collection No. 8739. Stock cultures are maintained on nutrient agar (Difco) slants and stored at 5° C. Transfers are made at monthly intervals. Two methods have been used to grow the organisms for the preparation of cell suspensions. By the first method the medium consists of 1 per cent Bacto peptone and 2 per cent agar in distilled water and dispensed in Kolle or Roux flasks. The agar surface is inoculated with a culture grown eighteen to twenty-four hours in 1 per cent peptone at 37° C. The inoculated flasks are incubated at 37° C. for sixteen to eighteen hours following which the growth is washed off with M/15 phosphate buffer at pH 6.0 using 5 to 10 ml. per flask. Care should be taken to avoid getting particles of agar in the suspension. The suspension is centrifuged for ten minutes at 3,000 r.p.m., washed once, and resuspended in the pH 6.0 buffer. The cell suspension is adjusted by diluting with buffer so that 1 ml. contains 0.20 to 0.25 mg. dry weight of cells. A 1:10 dilution of this adjusted suspension gives a reading on a Klett-Summerson photoelectric colorimeter, No. 54 filter, of 100 to 150 depending on the sensitivity of the individual instrument. Cell suspensions maintain good nitratase activity for a period of at least seven days when stored at 5° C.

By the second method the medium consists of a 1 per cent peptone solution instead of the agar medium. The culture is grown while maintaining vigorous aeration of the broth for sixteen to eighteen hours at 37° C. The cells are then washed and resuspended as above. This method is less time-consuming than the first and avoids the contamination of the bacterial suspensions with particles of agar.

From the Departments of Bacteriology and Physiology, University of North Carolina School of Medicine.
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B. *Nitrate Test.*—1. To a clean, 15 ml. conical centrifuge tube add the following:

a. 2 ml. of the nitrate-containing solution which has been diluted to a concentration of between 0.01 and 0.07 meq of nitrate per liter. Preliminary assays of various dilutions of the unknown will indicate the appropriate dilution required.

b. 1 ml. of M/15 sodium formate in M/15 phosphate buffer at pH 6.0.

c. 1 ml. of 2 per cent sodium para-aminohippurate. (Presumably other substances capable of forming a diazonium compound with nitrite could be used.)

d. 1 ml. of cell suspension.

2. Mix and incubate tubes in a water bath at 37° C. for thirty minutes.

3. Add 1 ml. of 1.2 N HCl and mix.

4. Centrifuge sufficiently to yield a clear supernatant (ten minutes at 3,000 r.p.m.).

5. To 4 ml. of the supernatant add 1 ml. of 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride or similar coupling reagent, mix well, and allow to stand for at least five minutes before determining colorimeter reading (540 filter). (In our experience the reagent blank has shown the same transmittance as water.)

6. From the standard curve (Fig. 1) calculate the concentration of the nitrate in the unknown.

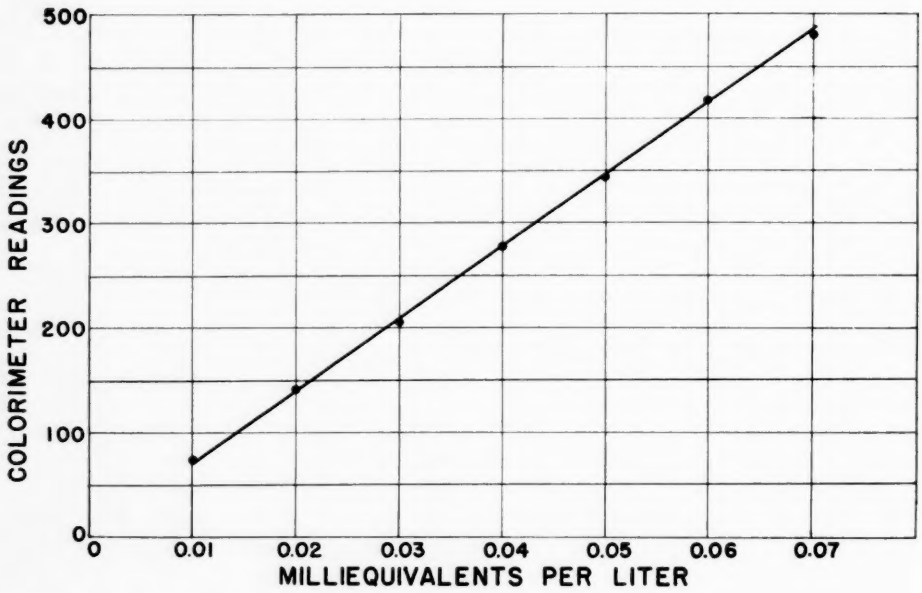

Fig. 1.—Typical standard curve for nitrate determination.

C. *Standard Nitrate Curve.*—Set up a standard curve for nitrate reduction using four or five known concentrations of sodium nitrate (reagent grade) to cover the effective range of 0.01 to 0.07 meq. per liter. Plot the colorimeter readings obtained by the above procedure against the nitrate concentrations. It is preferable to run a standard curve for each day's use of the cell suspension.

In order to check the nitratase activity of a given cell suspension, colorimeter readings obtained with known amounts of nitrate (standard curve) are compared with those obtained with equimolar solutions of sodium nitrite. To prepare such a control system, use 2 ml. of nitrite solution, 2 ml. of distilled water, 1 ml. of the para-aminohippurate solution and 1 ml. of 1.2 N HCl. Then to 4 ml. of this mixture add 1 ml. of the coupling reagent and take

colorimeter reading as above. In our experience the colorimeter reading of the nitrate solution is about 85 to 90 per cent of the reading obtained with an equimolar nitrite solution.

RESULTS

A typical standard curve for the reduction of nitrate to nitrite is shown in Fig. 1. Within the working limits of the colorimeter the curve is linear, except at concentrations under 0.01 meq per liter. It has been found preferable to dilute the sample to the working range prior to reduction rather than to dilute the colored solution at the end of the test. This is because concentrations of nitrate over 0.07 meq per liter may lower the proportion of nitrate reduced.

The use of more concentrated cell suspensions was tried. With heavy cell suspensions (more than three to four times that mentioned above), greater nitratase activity was observed, but at the same time there was an increased loss of nitrite so that the end result was a lower proportional recovery.

Application of this method to the determination of nitrate in plasma and urine has proved feasible.[2] Though determinations of nitrate in these fluids show more variations than is desirable, we have found this method more suitable to our purposes than any other we have tried. We have found it necessary to dilute plasma 1 to 10 and urine 1 to 100 in order to recover added quantities of nitrate by the method we have described. Whether the failures to recover from undiluted plasma and urine is due to alteration of pH or to other inhibitory factors remains to be determined. The plasma blank is small in the absence of hemolysis, but the urine blank (presumably nitrate of dietary origin) is often quite large and varies widely. In our experience dog urine has a negligible blank in 1 to 100 dilution but some of our human urine samples have shown an apparent nitrate content as high as 2.5 meq. per liter. Obviously the necessity of diluting plasma and urine reduces the sensitivity of this method as applied to these fluids.

TABLE I

NO_3 CONC. (MEQ/L.)	PLASMA RECOVERIES (PER CENT)	URINE RECOVERIES (PER CENT)
0.01	118, 103, 110, 90	114, 105, 102, 105, 116
0.03	97, 101, 92, 95	109, 110, 112, 101, 106
0.05	105, 103, 97, 97	108, 107, 103, 102, 103
0.07	99, 98	103, 106

Nitrate recoveries after adding measured amounts of $NaNO_3$ to human plasma diluted 1 to 10 with water and to human urine diluted 1 to 100 with water. The recoveries are expressed as percentages of the average of duplicate determinations on standard aqueous solutions analysed at the same time.

In Table I we show typical recoveries of added nitrate from diluted human plasma and urine expressed as percentages of simultaneous recoveries from aqueous solution.

The new method is obviously not suitable for nitrate determination with solutions containing compounds which might act as enzyme inhibitors.

SUMMARY

A method is described for the quantitative determination of nitrates with a sensitivity of approximately 0.01 meq per liter (0.085 mg. per cent of sodium

nitrate). The principle involved is that of enzymic reduction of nitrate to nitrite followed by a colorimetric determination of the nitrite from which by means of a standard curve the original nitrate concentration is calculated. The method is applicable for the determination of nitrates in body fluids.

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IDENTIFICATION OF ABNORMAL HEMOGLOBINS BY MEANS OF PAPER ELECTROPHORESIS

THEODORE H. SPAET, M.D.
SAN FRANCISCO, CALIF.

THE electrophoretic analysis of proteins has been a procedure requiring expensive and complex equipment, thus confining its application mainly to research institutions. Recently Durrum[1] has devised a simplified electrophoretic apparatus in which strips of filter paper replace the traditional glass cell. This apparatus can be constructed from common laboratory equipment, or can be purchased at extremely low cost.* Qualitative protein separations can be obtained readily from minute samples of test solutions. The validity of paper electrophoresis has been amply confirmed, and various modifications of the apparatus have been introduced to meet specific requirements.[2-4] Kunkel and Tiselius[5] made detailed investigations into the various physical factors involved, and worked out a method for quantitative estimation of the protein fractions.

The discovery by Pauling and his associates[6] that sickling of erythrocytes is associated with an abnormal hemoglobin of altered electrophoretic mobility has greatly enhanced the understanding of sickle-cell disease. Patients with the sickle-cell trait were found to have less than 50 per cent of the abnormal pigment, whereas those with sickle-cell anemia had the abnormal hemoglobin in almost pure form. A second type of hemoglobin having an electrophoretic mobility different from both the normal and sickle varieties has been described by Itano and others.[7, 8] When this type of hemoglobin was combined with normal hemoglobin in the red blood cells, no clinical disease was evident, although target cells were seen in the peripheral blood smear. When combined with sickle-cell hemoglobin, this second abnormal pigment was associated with a hemolytic syndrome resembling mild sickle-cell anemia in which splenomegaly was prominent. Most recently Itano[9] has reported yet another abnormal hemoglobin, which has an electrophoretic mobility identical to that of the sickle variety, but is considerably more soluble and is not capable of causing the erythrocyte to sickle. This pigment, too, was associated with anemia only when combined with sickle hemoglobin. Itano[9] has attempted to simplify the nomenclature of the hemoglobins by calling the normal, sickle, and the second (originally "type III") and third abnormal forms "a" "b," "c," and "d" respectively. All abnormal pigments appear to be genetically determined.

In addition, it has been demonstrated by both British and American workers[10, 11] that adult and fetal hemoglobin differ in their electrophoretic mobilities.

From the Department of Medicine, Stanford University School of Medicine, and the Department of Pediatrics, San Francisco County Hospital, San Francisco, Calif.

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*Dioptric Engineering Laboratories, 47 West 56th Street, New York, N. Y.

These findings are of especial interest inasmuch as Singer and associates[12] have reported a hemoglobin resembling that of the fetus to be present in patients with sickle-cell anemia and in certain other anemias.

It is evident that electrophoretic analysis of hemoglobin has clinical utility in addition to its value as a research tool for the study of sickle-cell disease and other anemias. An attempt was therefore made to determine the applicability of paper electrophoresis to the study of abnormal hemoglobins.

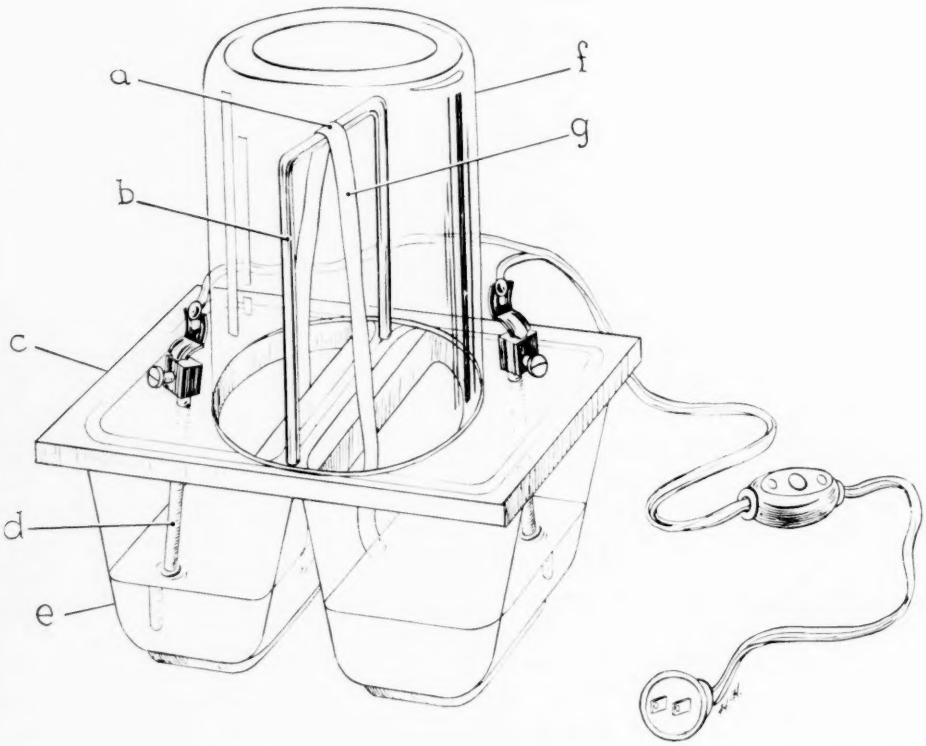

Fig. 1.—Apparatus for paper electrophoresis. (*a*) point of application of test solution; (*b*) glass rod; (*c*) plastic cover; (*d*) carbon electrode; (*e*) glass basin containing buffer; (*f*) glass cover; (*g*) filter paper strip.

METHODS

Electrophoretic apparatus.—Fig. 1 shows a diagram of the apparatus used. It is essentially similar to that described by Durrum.[1] The source of current was 110 volt DC obtained from the main hospital line with no modification. This potential resulted in a current of 0.1 milliamperes for each strip of paper. Similar voltage from dry cell batteries was found to be equally satisfactory. Paper strips were cut from squares of Whatman No. 1 filter paper to dimensions, 30 by 0.8 cm. These were washed with distilled water and dried before use.

Hemoglobin Solutions.—Oxalated blood was used. The erythrocytes were washed three times in normal saline. Washed, packed cells were hemolysed by freezing and thawing, and the resulting solution was diluted with five volumes of veronal buffer, pH 8.6. The mixture

was then centrifuged for an hour at about 20,000 r.p.m. in a Universal centrifuge with a high-speed attachment. The supernatant stroma-free hemoglobin solution was used without further modification.

Procedure.—(See Fig. 1.) The glass basins (*c*) were filled with veronal buffer, pH 8.6, to a level such that the distance from the fluid to the top of the glass rod (*b*) was about 14 centimeters. The fluid levels of the two basins were carefully equilibrated by siphoning. Paper strips were placed in position, and 0.003 c.c. of hemoglobin solution applied to the apex by means of a red blood cell counting pipette. The paper was then moistened with buffer almost to the apex, the remainder being allowed to wet by capillarity. The glass cover (*f*) was put in place, the system allowed to stabilize for ten minutes, and the current turned on for twenty-four hours. The paper strips were dried in an oven at 60° C. and stained for fifteen minutes in the following solution: bromphenol blue –0.1 Gm.; $HgCl_2$ –10 Gm.; 95 per cent ethanol qs ad 100 ml. This was followed by washing in tap water for about eight minutes, air drying at room temperature, and mounting.

A strip containing a known normal hemoglobin solution was always run simultaneously with the test solutions, and served as a control. Although there was some variation in the migration of the control from day to day, simultaneously run pairs of controls always showed the same migration behaviour.

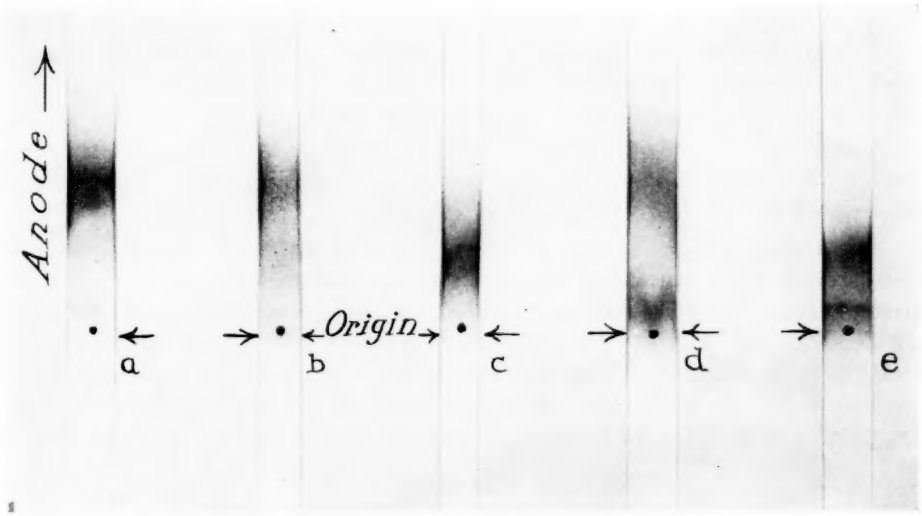

Fig. 2.—Electrophoretic patterns of normal and abnormal hemoglobins. *a*, Normal (aa); *b*, sickle-cell trait (ab); *c*, sickle-cell anemia (bb); *d*, "heterozygous" type "c" (III) (ac); *e*, Type "c" plus sickle (bc).

RESULTS

Fig. 2 shows typical patterns illustrating various combinations of hemoglobin abnormalities. It can be seen that in respect to distance of migration toward the anode, normal hemoglobin travels the farthest, type "b" (sickle) to an intermediate position, and type "c" (III) least. "Heterozygous" forms are clearly shown. The hemoglobins from patients with Mediterranean anemia, ovalocytosis, and congenital spherocytosis showed normal migration patterns. In the hemoglobin of umbilical cord blood it was not possible to resolve bands of fetal and adult pigment by the present technique.

DISCUSSION

The simplicity and economy of paper electrophoresis render this tool readily available for general clinical medicine. Its success in the identification of abnormal hemoglobins makes it a valuable method in the study of certain anemias. A most obvious application is the distinction between sickle-cell anemia and intercurrent anemia complicating sickle cell trait. Although the presence of alkali-resistant hemoglobin in sickle cell anemia in contrast to its absence in the trait has been helpful,[12] the finding of alkali-resistant hemoglobin in other unrelated anemias lowers the specificity of this test. The presence of a single band in the "b" (sickle) position of the electrophoretic pattern is indicative of sickle-cell anemia, whereas bands in both the "a" (normal) and "b" positions reveal the trait. Unfortunately, the present technique is not suited to the study of the role of alkali-resistant hemoglobin in the pathogenesis of sickle-cell disease. This pigment has common properties with fetal hemoglobin, and may be identical to it.[12] However, special methods are required for its electrophoretic separation; and such separation has not been accomplished on paper.

The discovery of type "c" (III) hemoglobin has introduced an additional etiological factor for target cells in the peripheral blood, and its combination with "b" (sickle) hemoglobin is the source of a new hemolytic syndrome. These abnormalities can best be diagnosed by electrophoretic methods, as can the recently reported syndrome resulting from the combined sickle and Mediterranean anomolies.[13] As new hemoglobin variants are discovered, the utility of electrophoresis will be correspondingly extended.

Mass studies with the present technique could be of considerable value in the clarification of sickle-cell disease as an anthropological problem. The reported paucity of sickle-cell anemia in parts of Africa where there is a high incidence of the trait has been recently disputed.[14] In Greece many cases of sickling in a primarily Caucasian population have stimulated the suggestion that the sickle and Mediterranean anomolies may be genetically related.[15] Electrophoretic studies should be of great interest in these situations.

SUMMARY

By means of paper electrophoresis a simple and economical method is available for the identification of certain abnormal hemoglobins. Examples are presented of "a," "b," and "c" hemoglobins in various combinations.

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AN ACCUMULATING IMPULSE COUNTER

AKIRA HORITA, M.S., AND JAMES M. DILLE, PH.D., M.D.
SEATTLE, WASH.

IN PHARMACOLOGY and physiology laboratory investigation the need frequently arises for comparative counts of repetitive events such as the heart rate or respiratory rate and for the counting of drops such as is done in measuring the outflow from the kidney or from secretory glands. It is usually comparatively simple to convert such recurring events into electrical impulses. Generally these impulses are led to an electromagnetic counter or to a signal magnet writing on a smoked paper kymograph. In either case the labor involved

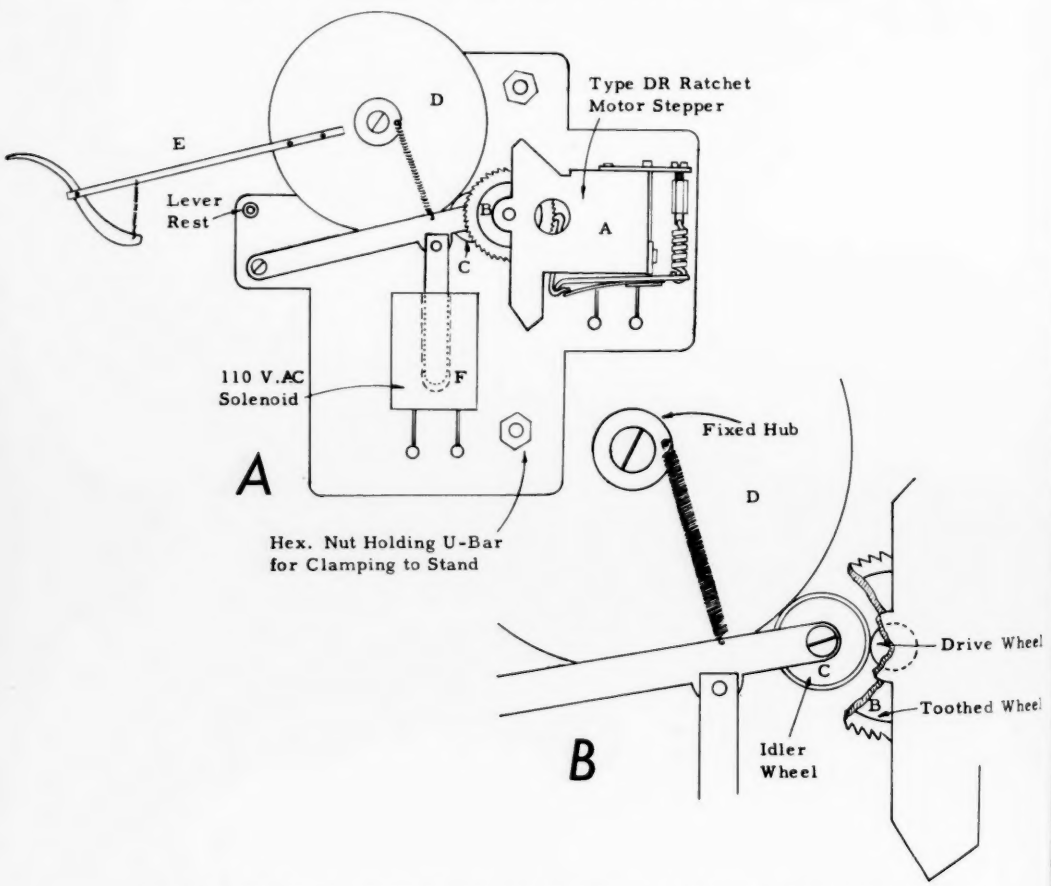

Fig. 1.—Mechanism of accumulating impulse counter. *A*, General arrangement of parts. *B*, Detail of idler wheel which transmits movement of ratchet motor stepper *A* to wheel *D* and recording lever E.

From the Department of Pharmacology, School of Medicine, University of Washington.
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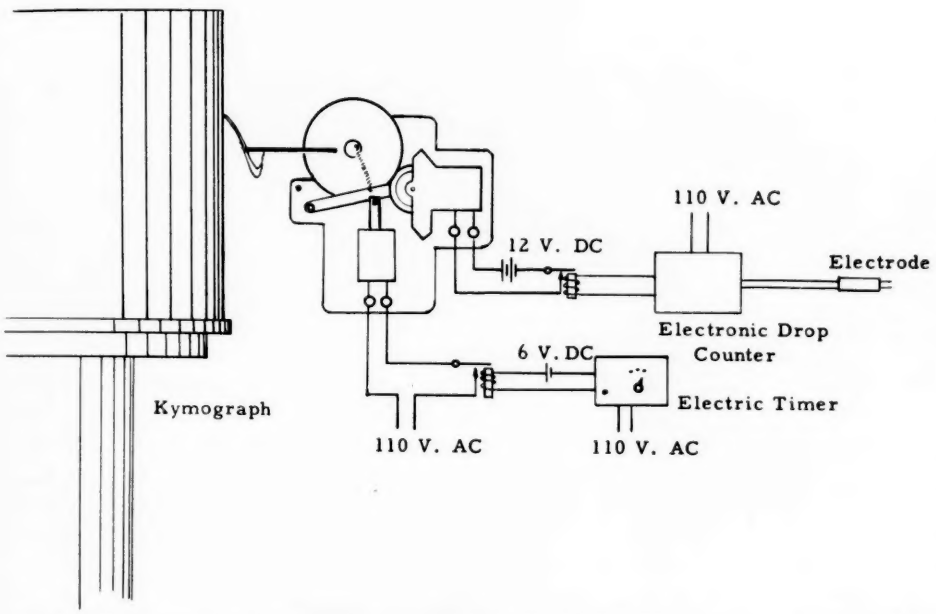

Fig. 2.—Diagrammatic arrangement of accumulating impulse counter used as a drop recorder.

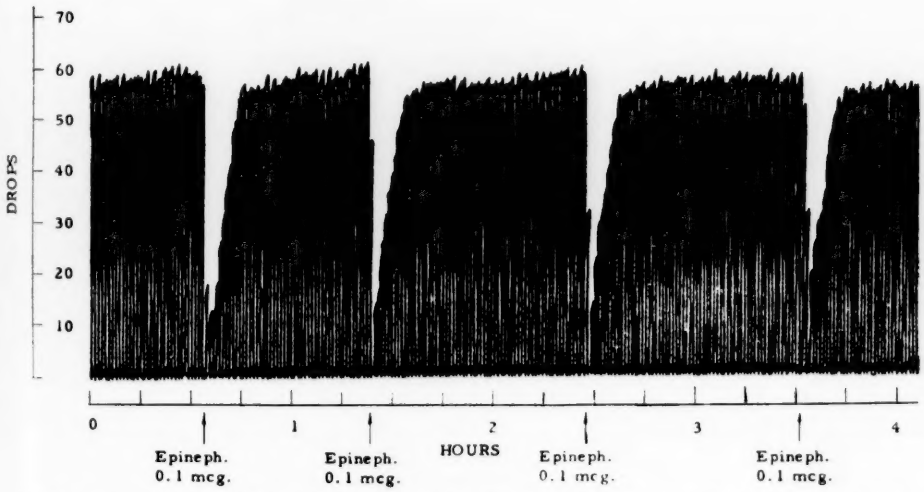

Fig. 3.—Record produced by the accumulating impulse counter of perfused rabbit ear showing vasoconstrictor effect of epinephrine.

in converting such records from long experiments into readily understandable tables or graphs is quite great. The desirable solution would be a means of producing a graphical representation directly. Various devices have been described for this purpose by Gaddum and Kwiatkowski,[1] Junkmann,[2] Fleisch,[3] Thorpe,[4] and Clementz and Ryberg.[5] Most of these involve some means of producing a vertical line on a smoked paper kymograph the height of which is indicative of the number of events occurring. A final record is of great value because its end result is essentially a bar graph the abscissa of which represents time while the ordinate represents the frequency of the event. The device described in this paper produces such a record and has the advantage of incorporating into it a ratchet relay and solenoid which are relatively inexpensive and effective.

Fig. 1 shows the general construction of the device. The essential part of the equipment is a ratchet motor stepper* "*A*". This device operates in such a manner as to rotate a toothed wheel "*B*" one notch for each electrical impulse reaching the electromagnet. The rotatory movement of the axis is transmitted through a rubber idler wheel "*C*" to a large freely rotating wheel "*D*" which carries a writing lever "*E*" so designed as to write a vertical record at right angles to the tangent of the kymograph drum. At suitable intervals, usually a minute, the rubber idler wheel is disengaged by means of a solenoid "*F*" operated from an interval timer. This disengagement of the idler wheel permits the writing lever to return by gravity to a base line.

We have found this device particularly suitable for recording the flow of perfused rabbit ears, and have produced continuous records for as long as twelve-hour periods. Arrangement for such recording is seen in Fig. 2, where falling drops making contact between two electrodes trigger an electronic drop counter of the type described by Brown and associates[6] which in turn actuates a relay operating the ratchet motor stepper.

Fig. 3 shows the type of record produced by this arrangement. The effect of a vasoconstrictor agent such as epinephrine may be perceived immediately by inspection, and the actual rates per minute can be determined by use of suitable scales for the ordinates and abscissa.

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*Type DR ratchet motor stepper, part of SCR 522 VHF tuning mechanism. Operating voltage 12 v. DC. Obtained from Herbach and Rademan, Inc., 1204 Arch Street, Philadelphia 7, Pa.